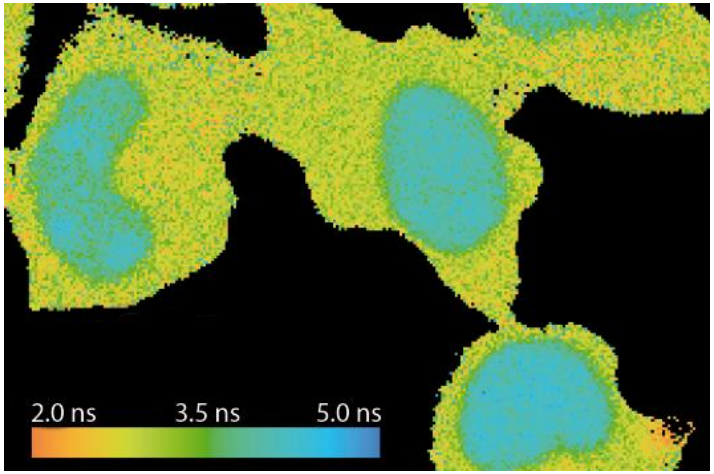

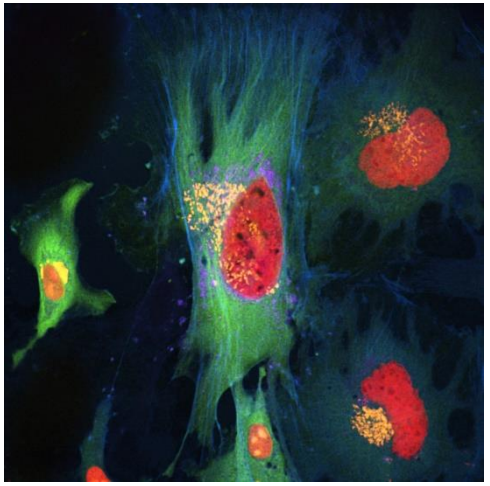

Fluorescent proteins for multiparameter imaging of live cells and animals

Konstantin Lukyanov
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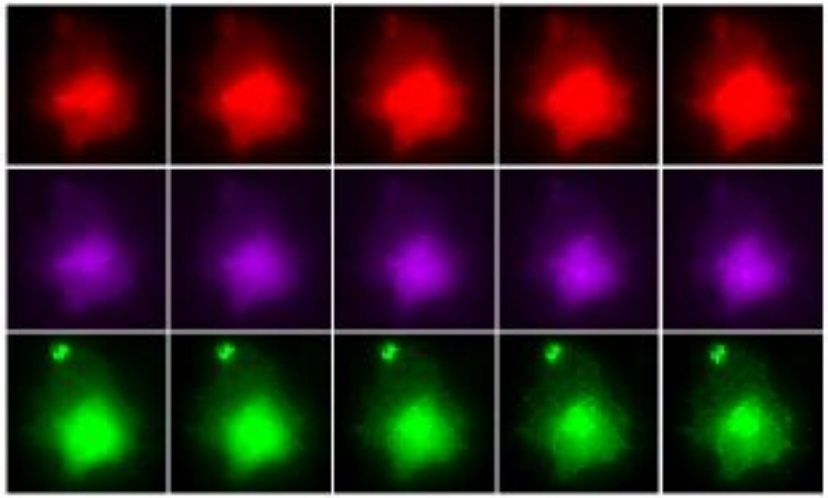
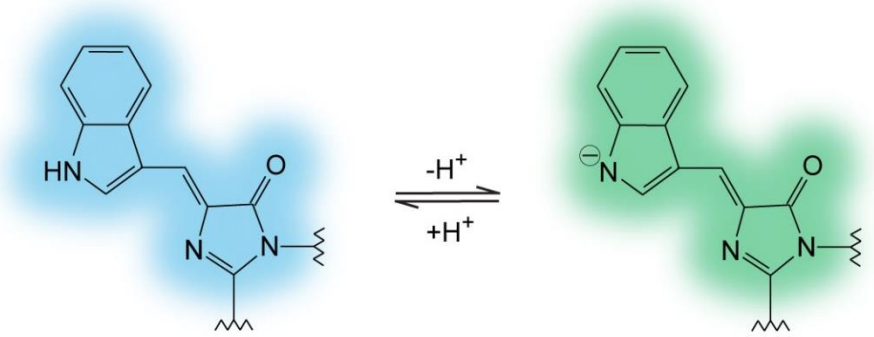
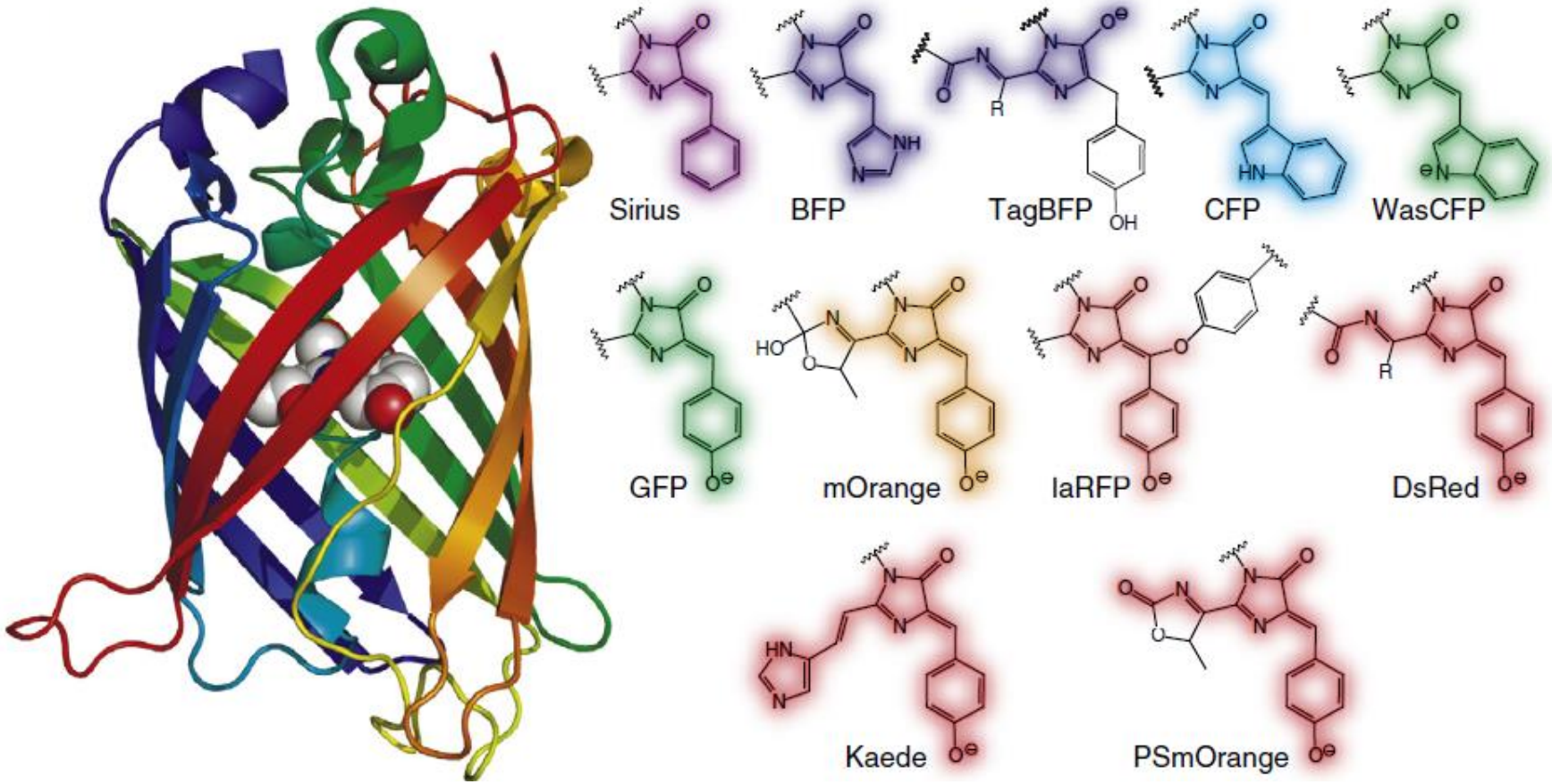
Institute of Bioorganic Chemistry
Moscow, Russia



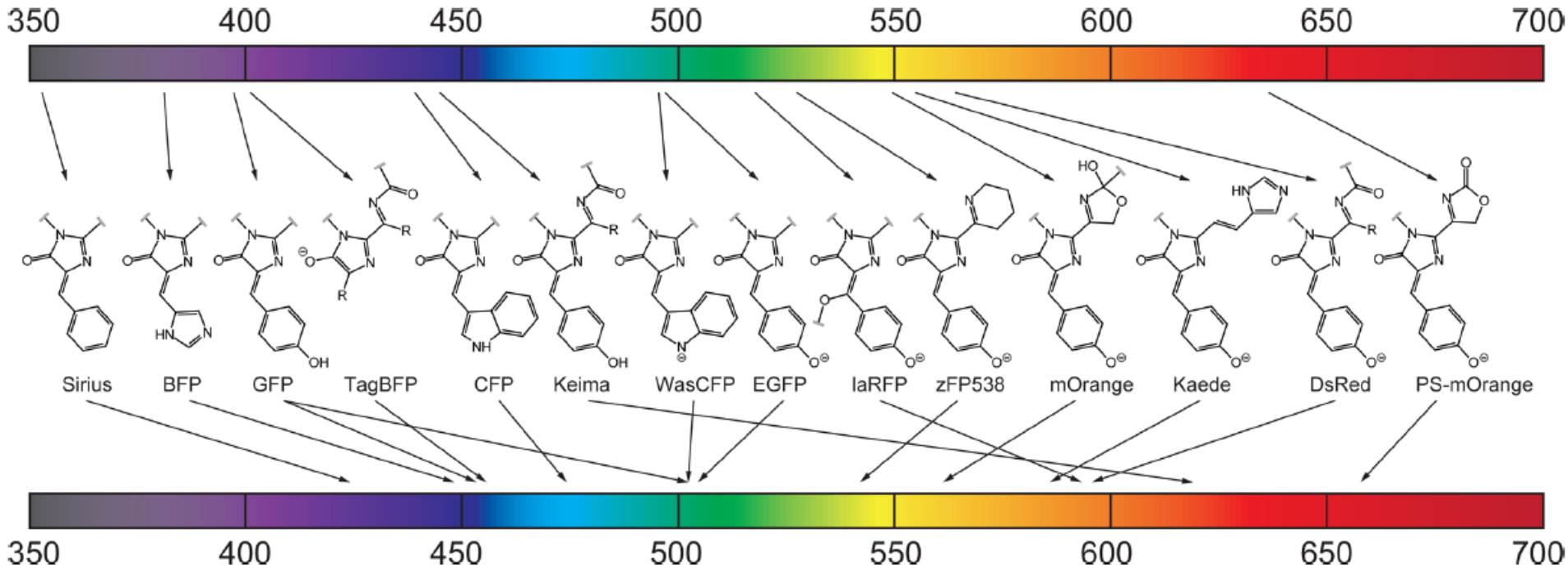


Fluorescent proteins of GFP family

- Fully genetically encoded fluorescent probes (only O_2 is required)
- Interesting biochemistry, photochemistry and photophysics

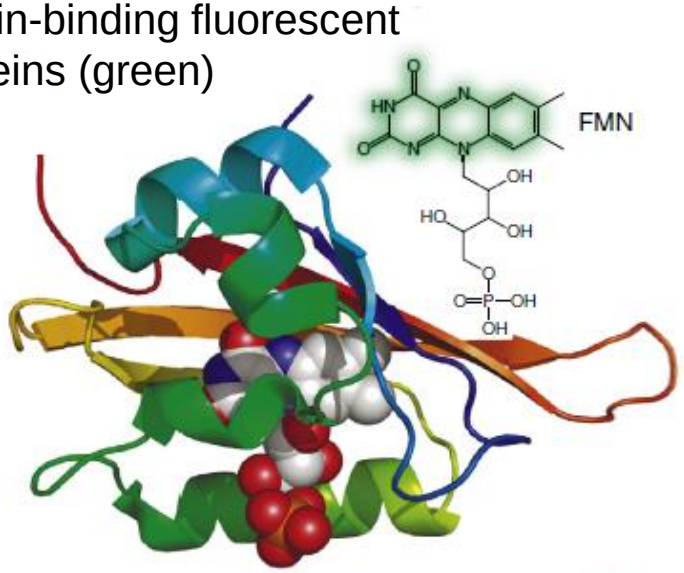


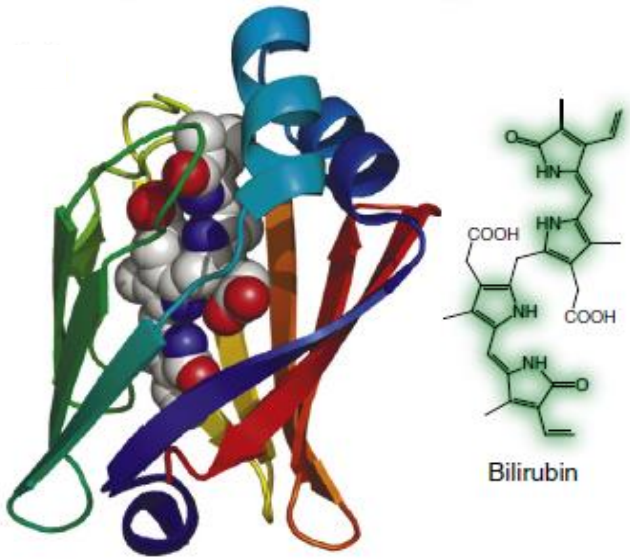
Fluorescent proteins of GFP-like proteins: chemically distinct chromophores produce a variety of colors



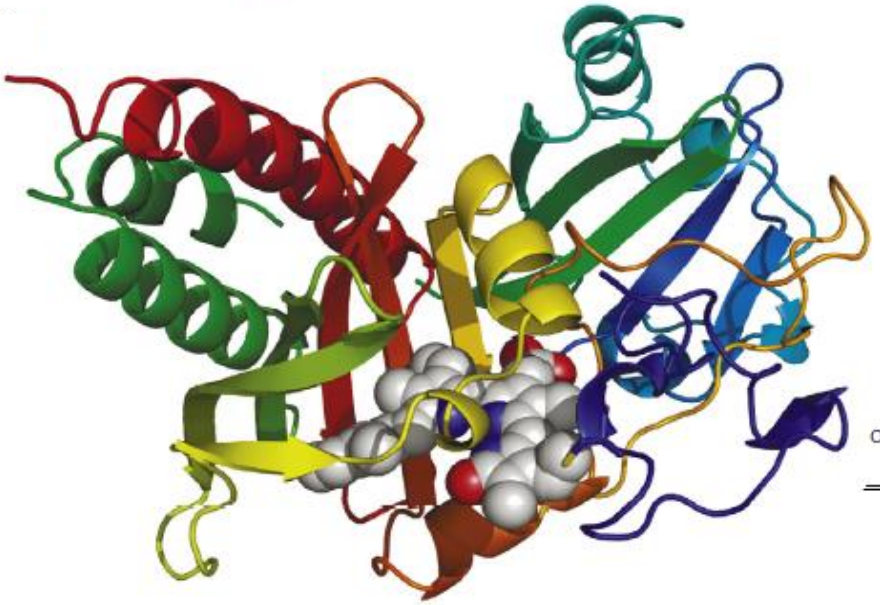
"Genetically encoded" fluorescent proteins that bind endogenous cofactors

Flavin-binding fluorescent proteins (green)

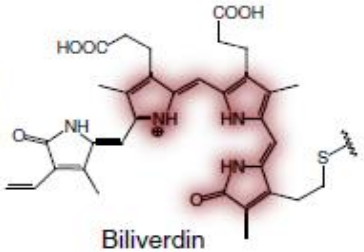




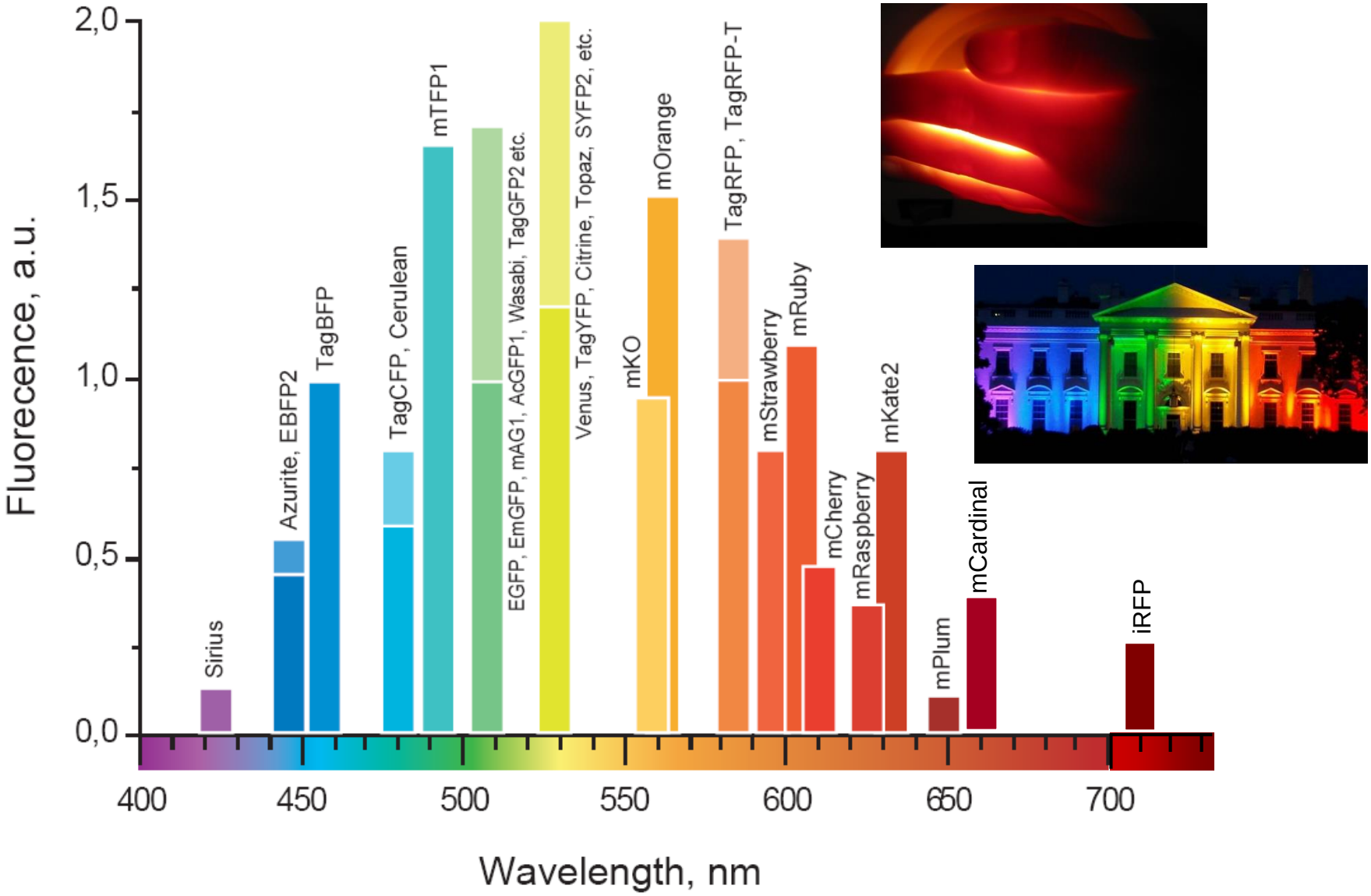
Bilirubin-binding fluorescent protein UnaG (green)



Biliverdin-binding fluorescent proteins (far-red – near infrared)



Spectral diversity of fluorescent proteins



Whole-body imaging with far-red fluorescent proteins

SCIENTIFIC REPORTS

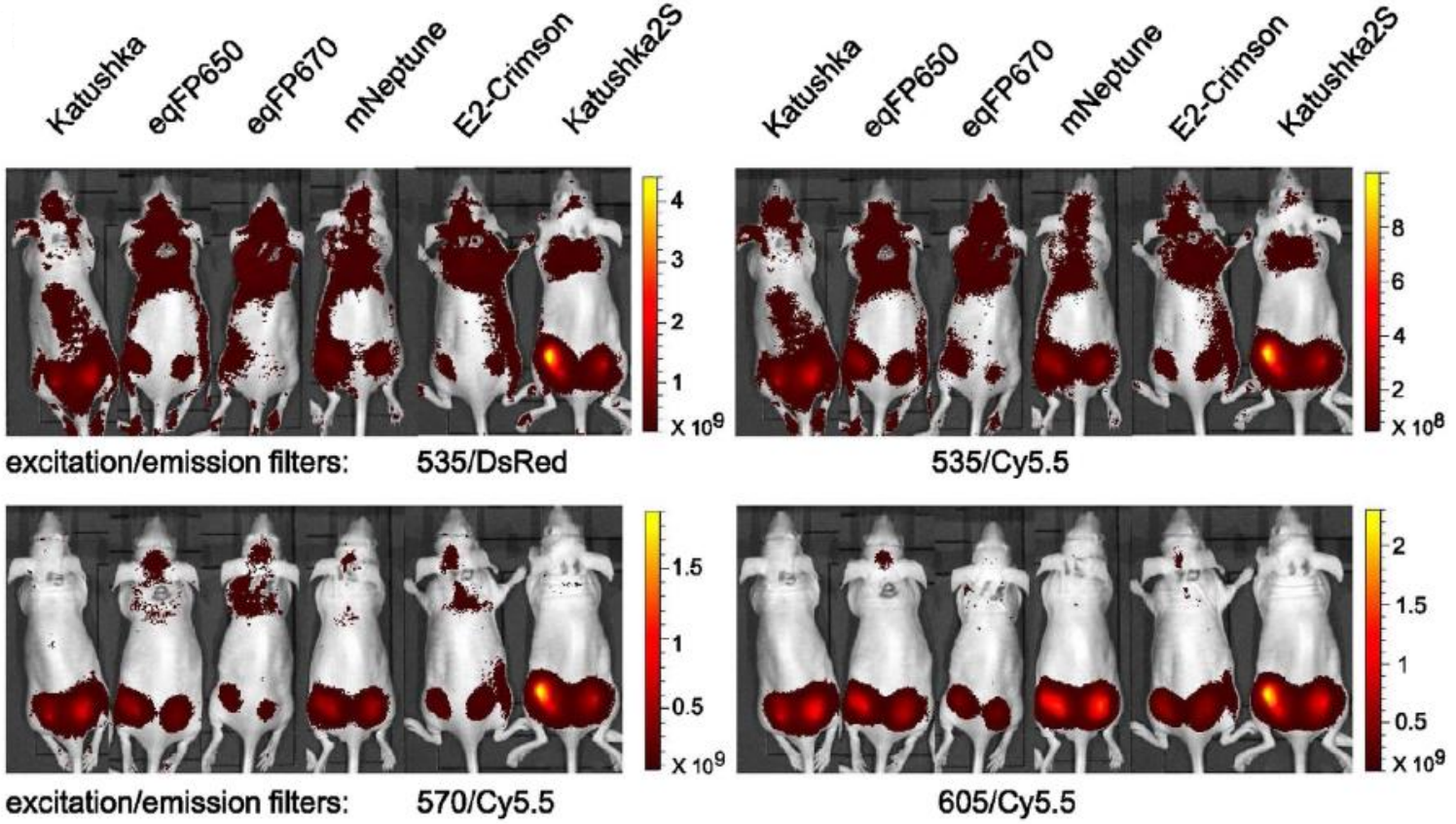
OPEN

Comparative study reveals better far-red fluorescent protein for whole body imaging

Received: 21 August 2014
Accepted: 08 April 2015
Published: 02 June 2015

K.E. Luker[1,*], P. Pata[2,*], I.I. Shemiakina[3,4,*], A. Pereverzeva[3,*], A.C. Stacer[1], D.S. Shcherbo[3,4], V.Z. Pletnev[3], M. Skolnaja[2], K.A. Lukyanov[3,5], G.D. Luker[1], I. Pata[2] & D.M. Chudakov[3,6,7]

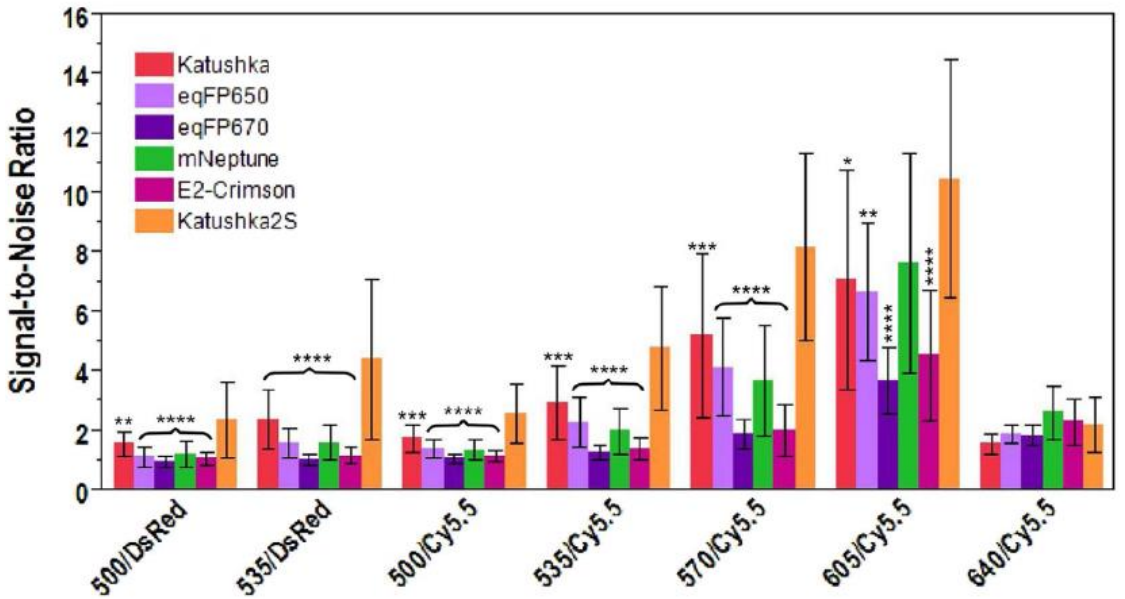
	eqFP670	E2-Crimson	mNeptune	eqFP650	Katushka	Katushka2S
Excitation peak (nm)	605	605	599	592	588	588[b]
Emission peak (nm)	670	646	649	650	635	633[b]
Fluorescence quantum yield	0.06	0.12	0.18	0.24	0.34	0.44[b]
Molar extinction coefficient ($M^{-1}\cdot cm^{-1}$) at excitation maximum	70,000	58,500	57,500	65,000	65,000[b]	67,000[b]
Brightness (a.u.)[a]	4,200	7,080	10,350	15,600	22,100	29,480[b]



IVIS Lumina II fluorescence images of nude mice injected into the gluteal muscle (5 mm depth) with HEK293FT cells ($5x10^6$) transiently expressing FPs together with IRES-driven luciferase.
Prior to injection, cells were normalized for transfection efficiency with luciferase activity.

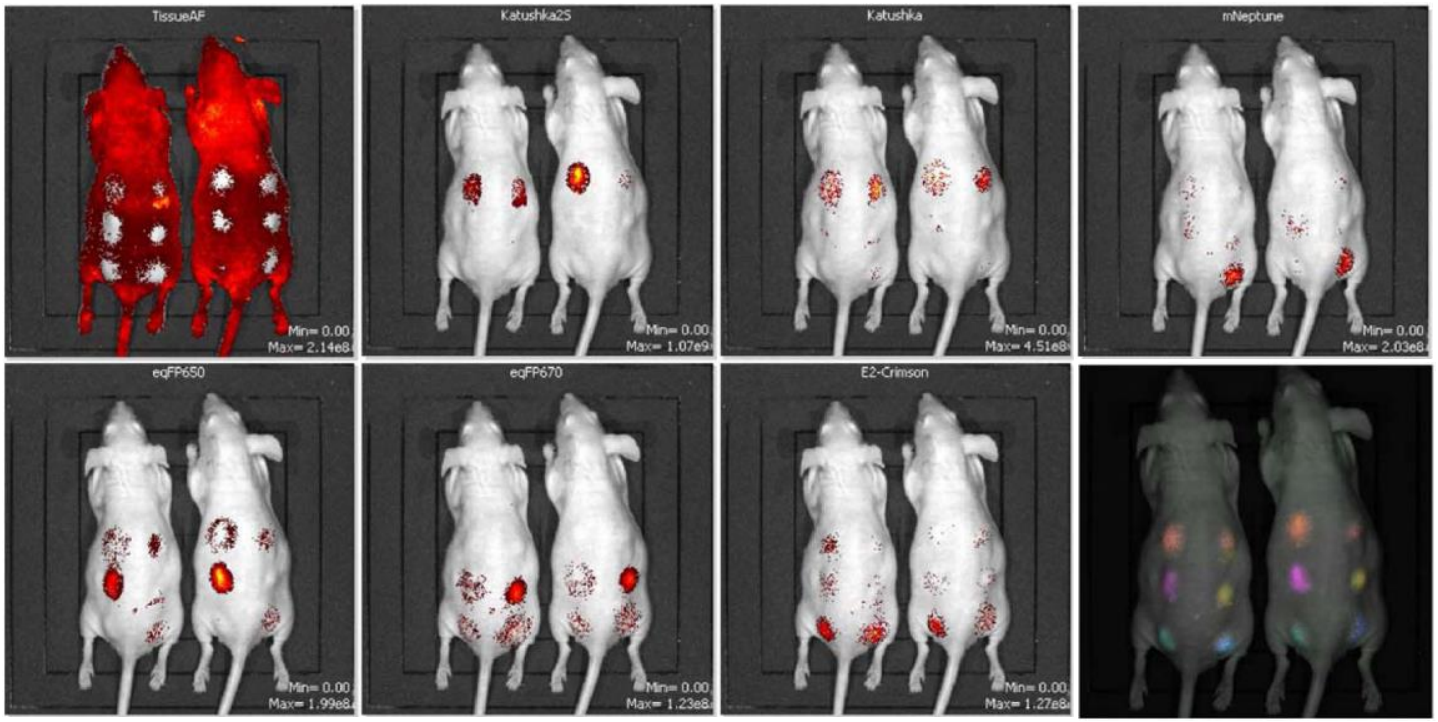
Comparison of signal-to-noise ratios of far-red FPs

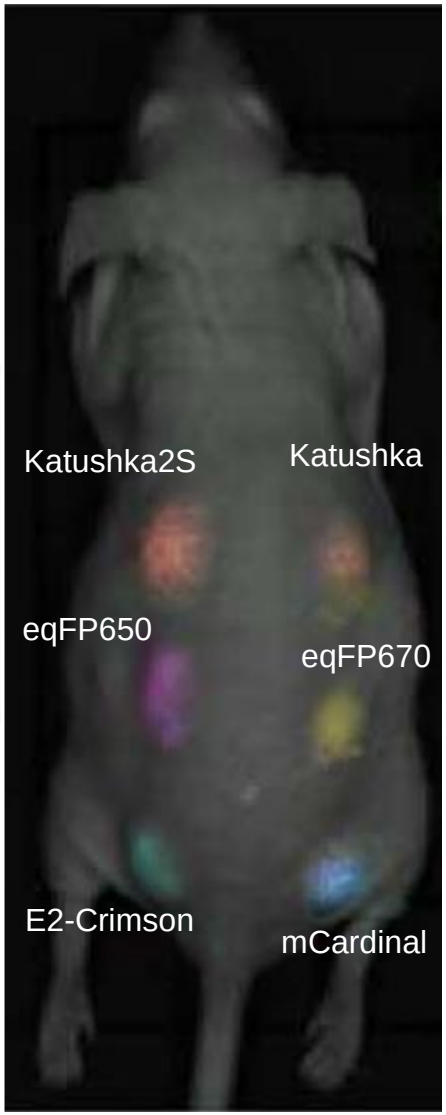
- IVIS Lumina II.
- Nude mice injected into the gluteal muscle, ~5 mm depth.
- HEK293FT cells (5x10^6) transiently expressing FPs together with IRES-driven luciferase.
- Normalization for transfection efficiency using luciferase activity.



Excitation, nm	500	535	500	535	570	605	640
Emission filter	DsRed		Cy5.5				
Protein	**Signal-to-noise-ratio**						
E2-Crimson	1.1	1.2	1.2	1.4	2.0	4.6	2.3
eqFP650	1.2	1.6	1.4	2.3	4.1	6.7	1.9
eqFP670	1.0	1.1	1.1	1.3	1.9	3.7	1.9
Katushka	1.6	2.4	1.8	3.0	5.2	7.1	1.6
Katushka2S	2.4	4.4	2.6	4.8	8.2	10.5	2.2
mNeptune	1.2	1.6	1.4	2.0	3.7	7.7	2.6

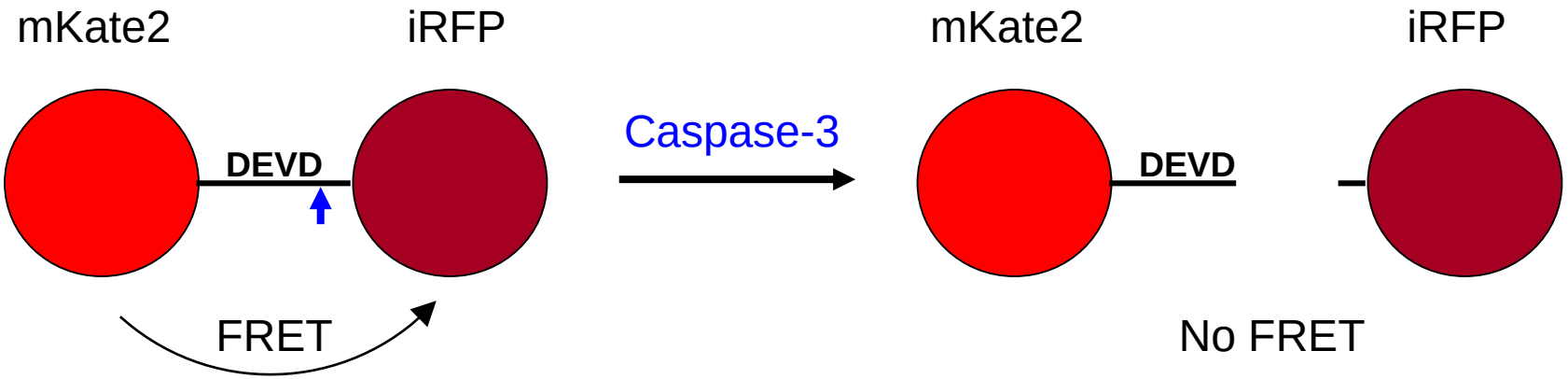
Multicolor imaging with far-red FPs using spectral unmixing

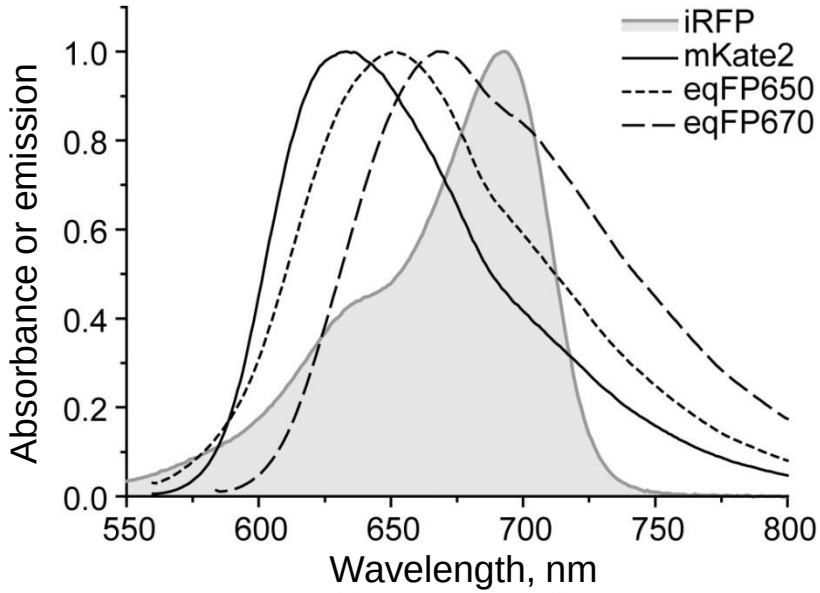




FP-expressing HEK293FT cells ($2.5x10^6$) were engrafted subcutaneously into the same mice and imaged using IVIS Lumina II.

Far-red FRET sensor for caspase-3 activity



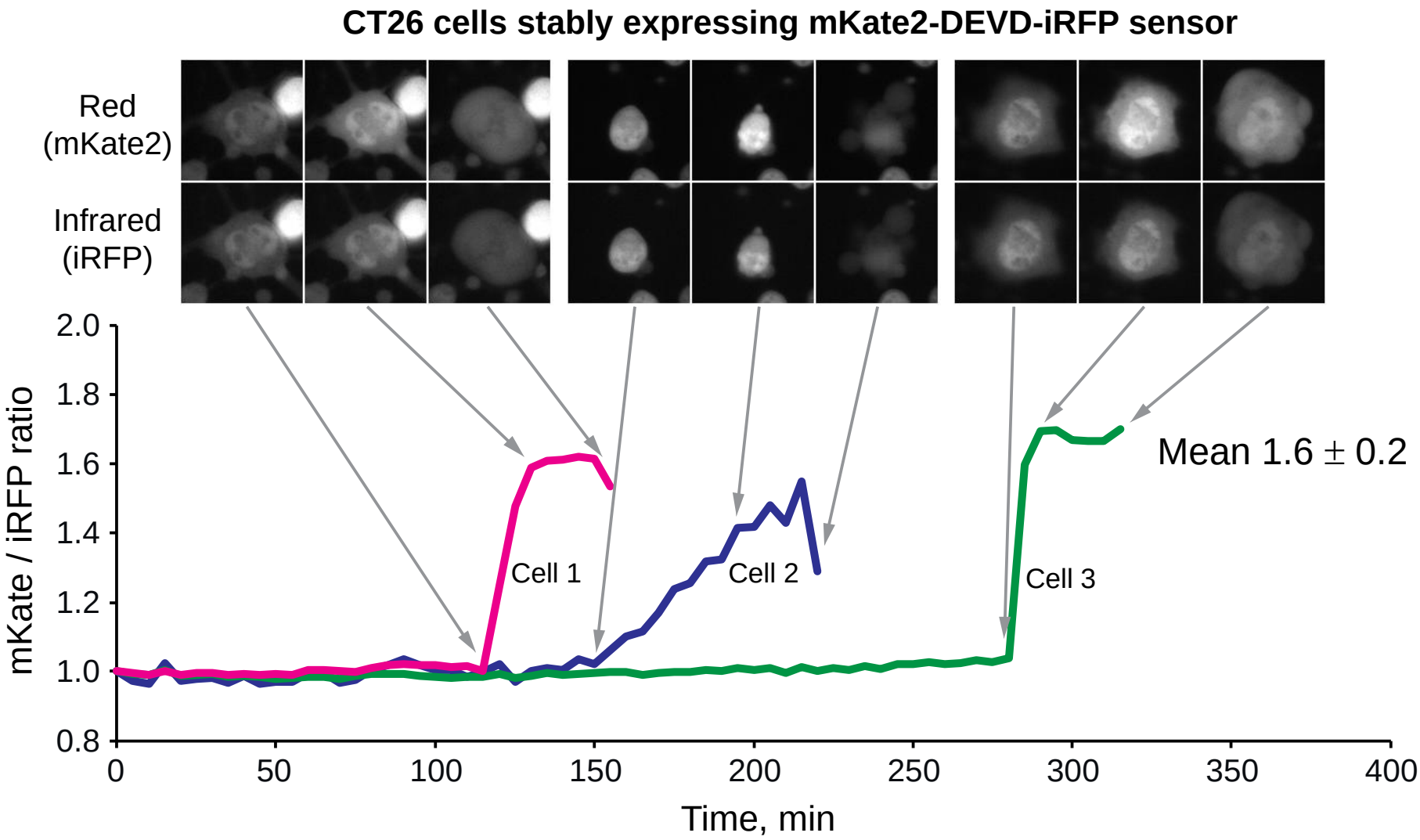


Potential advantages:

- Better light penetration in whole-body imaging

- Free channels from blue to orange for multicolor imaging (all GFP-based fusions and sensors can be used)

Caspase-3 activation during staurosporine-induced apoptosis



Zlobovskaya et al.,
Biotechniques, Feb 2016.

Multiparameteric imaging: caspase-3 activation + Bax translocation

Staurosporine

↓

Bak Up-regulation

Bax & Bcl-XS Translocation

EGFP-Bax

↓

Cytochrome C ↑

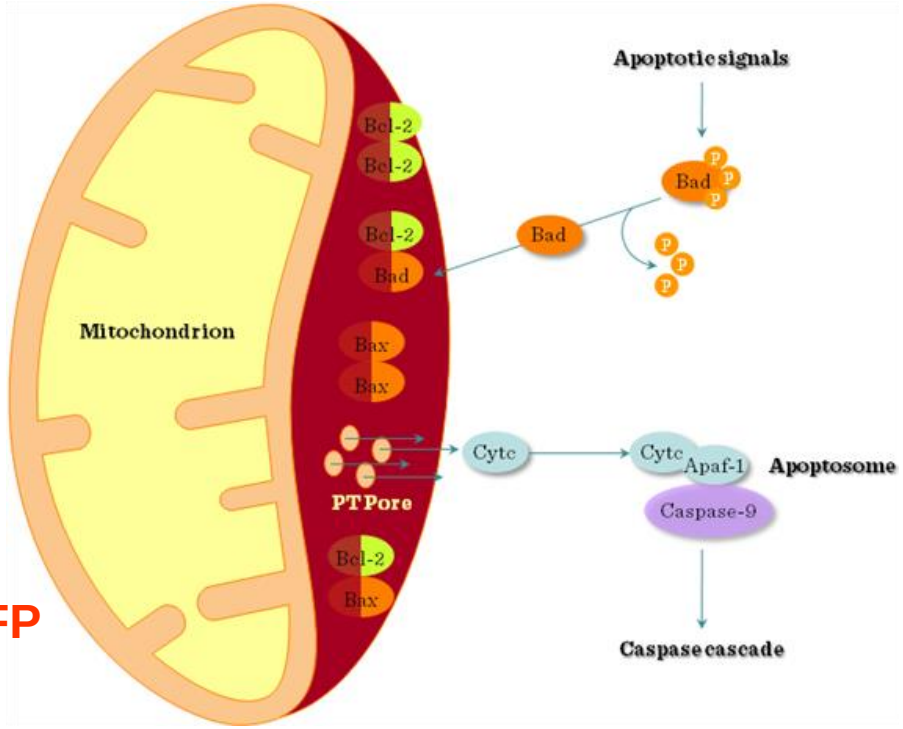
↓

Caspase-3 Activity ↑

mKate2-DEVD-iRFP

↓

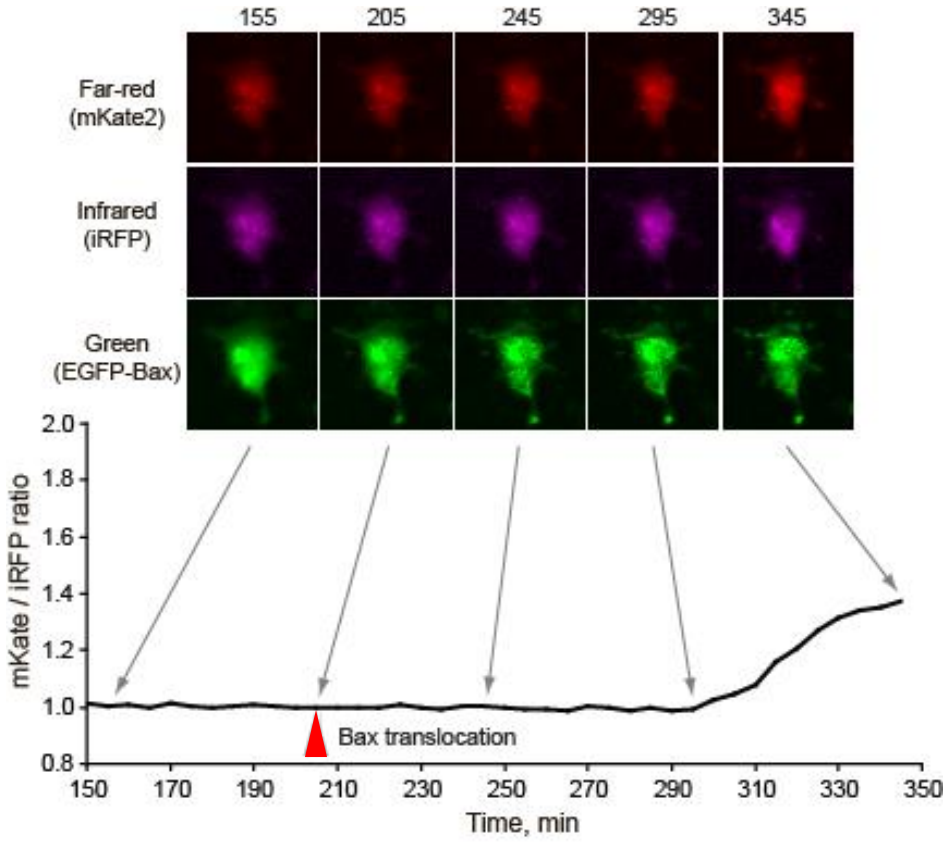
Apoptosis



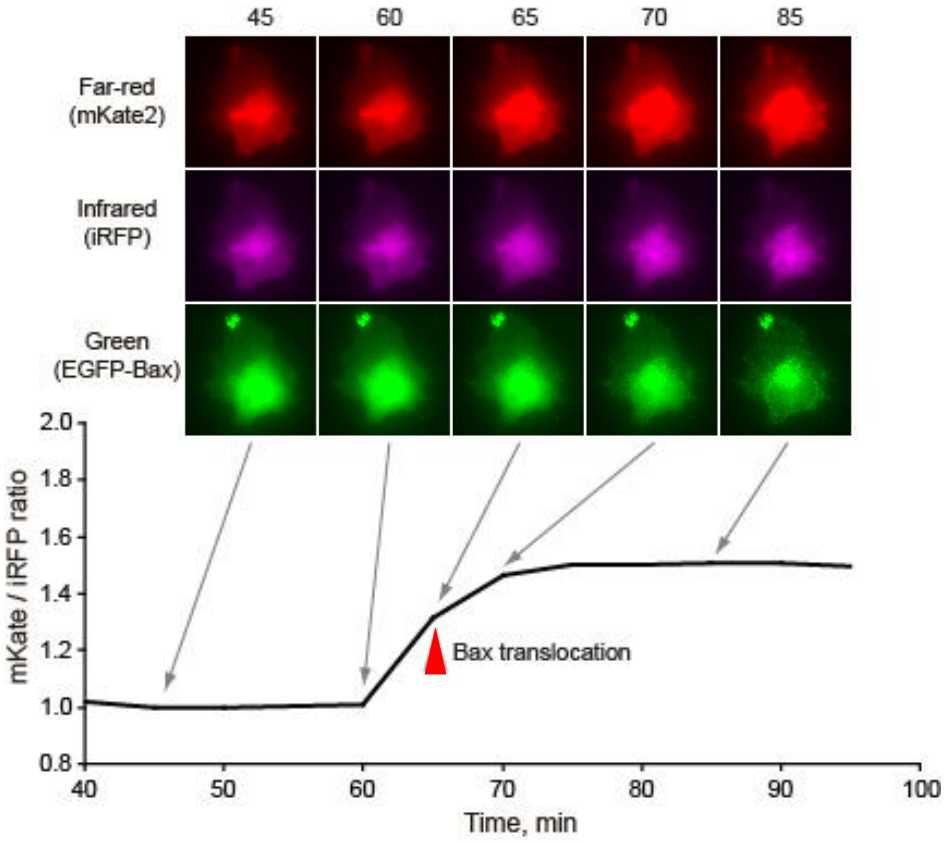
Cell Death and Differentiation (2004) 11, 512–526.

http://www.tankonyvtar.hu/hu/tartalom/tamop425/0011_1A_Jelatvitel_en_book/ch03s04.html

Caspase-3 activation + Bax translocation during staurosporine-induced apoptosis

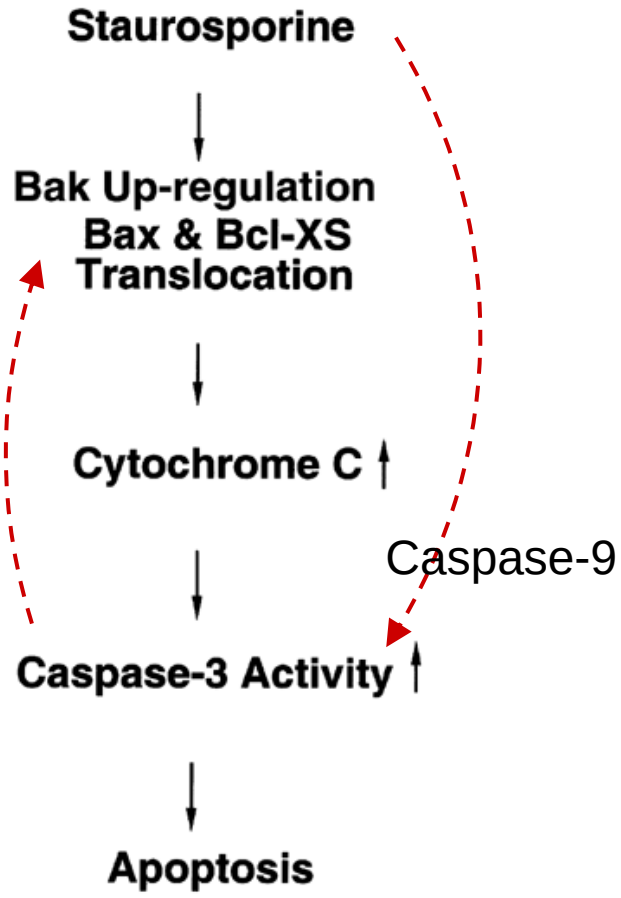


Early Bax translocation (10% of the cells)



Late Bax translocation (90% of the cells)

Multiparameter imaging: caspase-3 activation + Bax translocation



The FASEB Journal • Research Communication Vol. 25 September 2011

Triggering of a novel intrinsic apoptosis pathway by the kinase inhibitor staurosporine: activation of caspase-9 in the absence of Apaf-1

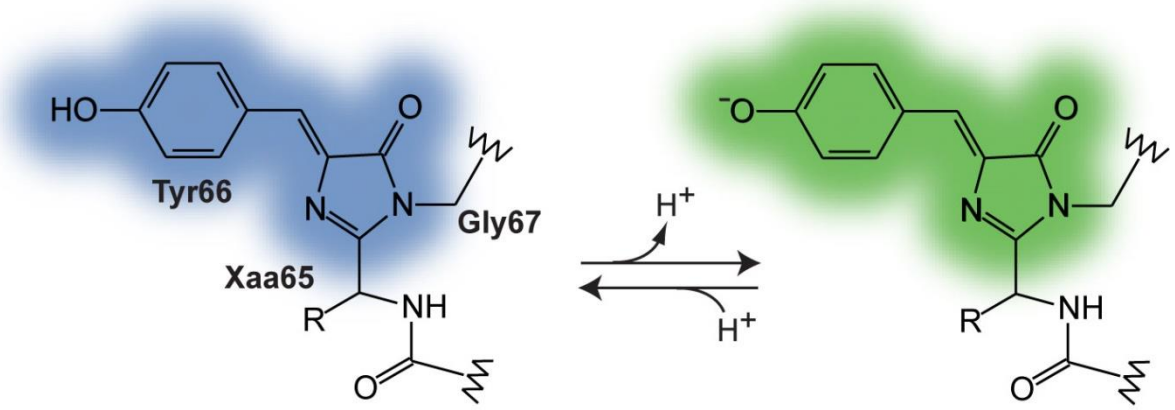
Joachim Manns,*,[1] Merle Daubrawa,*,[1] Stefan Driessen,*,[2] Florian Paasch,* Nadine Hoffmann,* Antje Löffler,*,[2] Kirsten Lauber,*,[3] Alexandra Dieterle,* Sebastian Alers,* Thomas Iftner,† Klaus Schulze-Osthoff,‡ Björn Stork,*,[2,4] and Sebastian Wesselborg*,[2,4,5]

*Department of Internal Medicine I, †Division of Experimental Virology, Institute for Medical Virology and Epidemiology of Viral Diseases, and ‡Interfaculty Institute for Biochemistry, University of Tübingen, Tübingen, Germany

Cell Death and Differentiation (2004) 11, 512–526.

Fluorescent proteins with anionic tryptophane-based chromophore

Protonation-deprotonation is a common feature of Tyrosine-based green and red chromophores



Chromophore ionization is used in:

• Photoactivatable fluorescent proteins – PA-GFP, PS-CFP, Dronpa, *etc*.

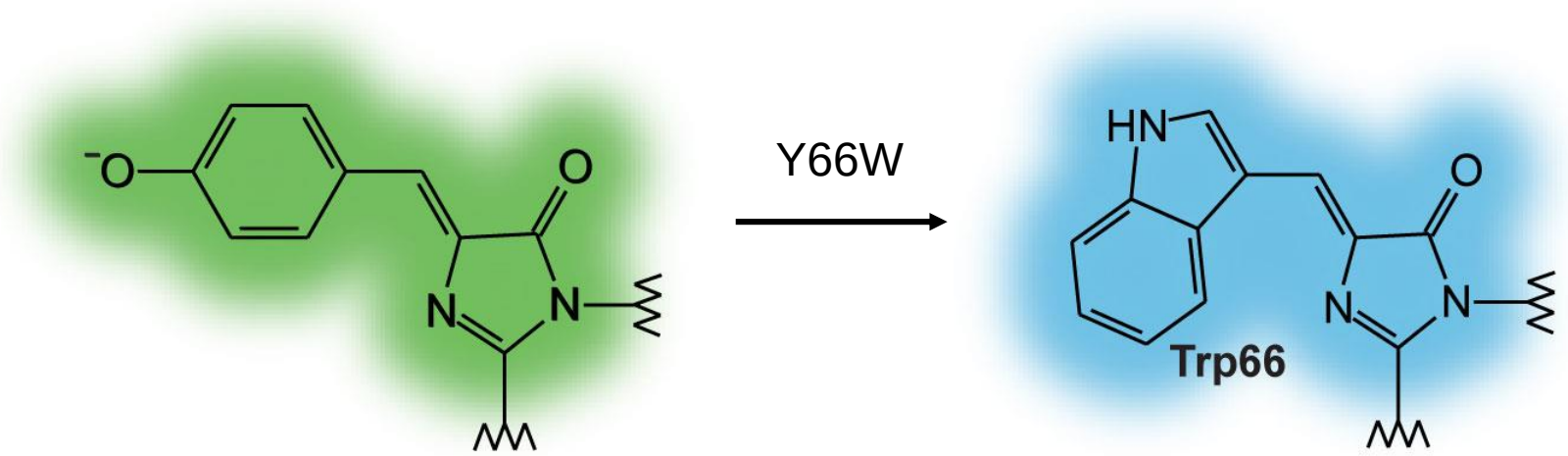
• Sensors – Pericams, GCaMPs (Ca^{2+}), pHluorins**,** deGFP (pH), roGFP (redox), HyPer (H_2O_2), *etc*.

• Large Stocks-shift fluorescent proteins – Sapphire, Keima, LSS-mKate, *etc*.

Cyan Fluorescent Proteins: chromophore's Tyr mutated to Trp

Cyan FPs (ECFP, Cerulean, mTurquoise) with chromophore-forming Trp66 are widely used for multicolor labeling and FRET.

No charged states of CFP chromophore have been described.

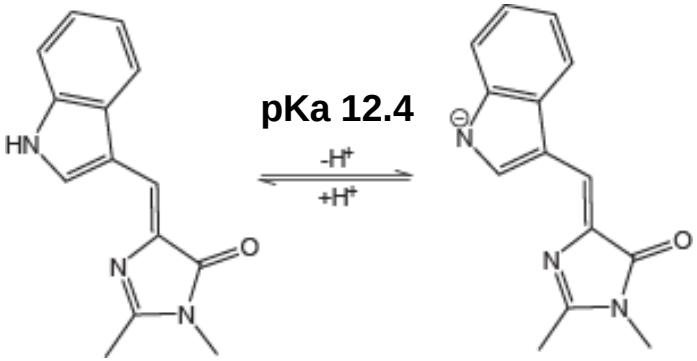


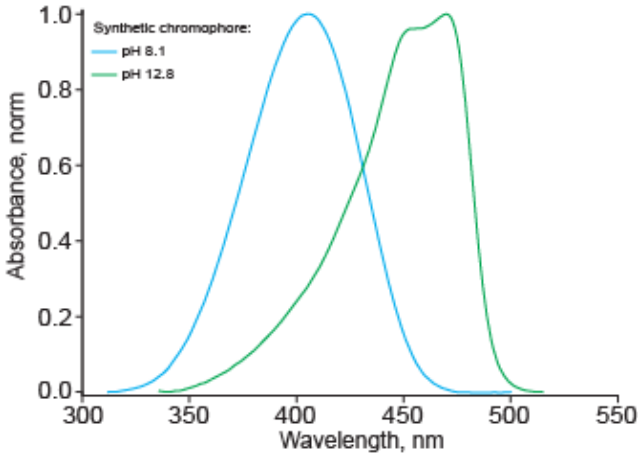
GFP chromophore

CFP chromophore

Can we create fluorescent protein with anionic tryptophan-based chromophore?

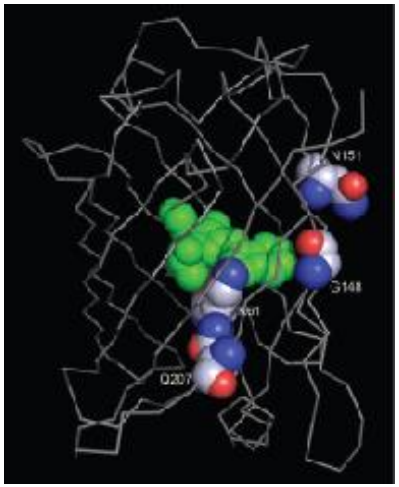
Study of synthetic CFP chromophore



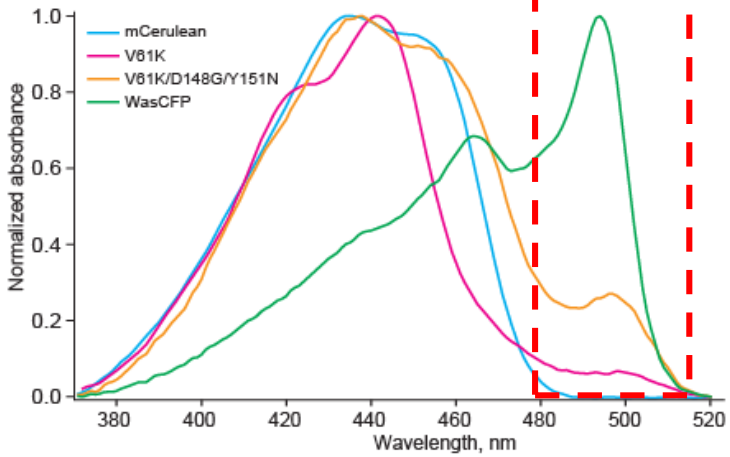


Molecular evolution of mCerulean:

Lys/Arg near Trp66 + random mutagenesis

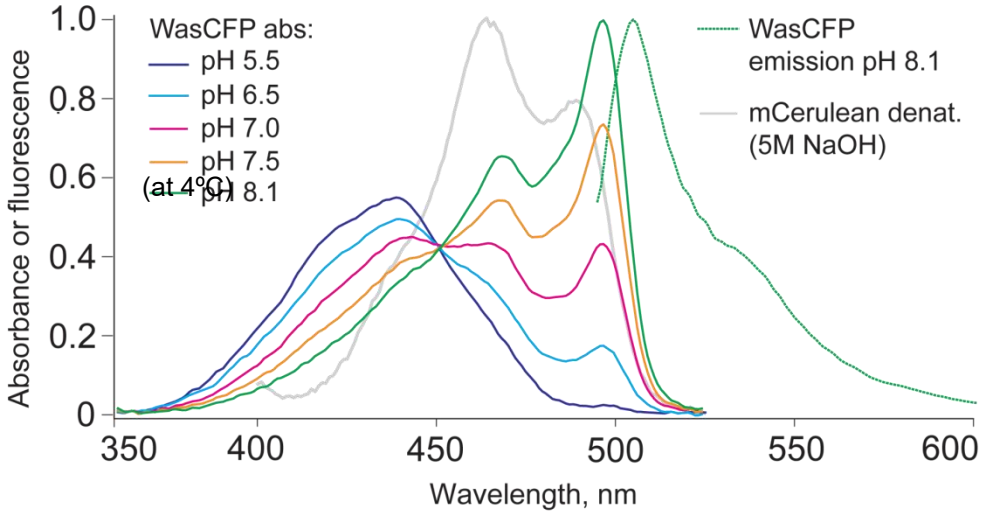


New form!



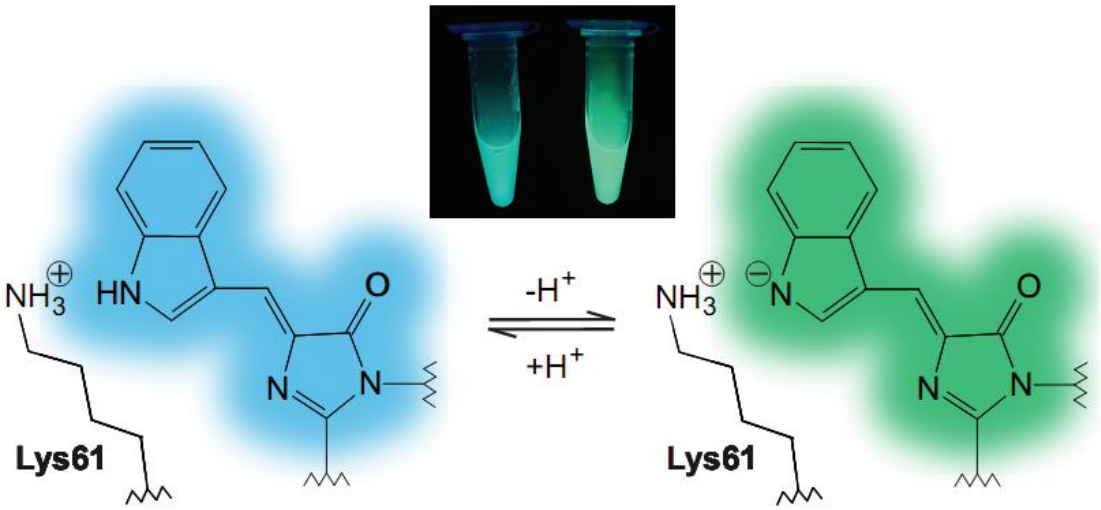
Sarkisyan el al., Sci. Rep. 2012

WasCFP (W in anionic state) – the first FP with anionic Trp66 in chromophore



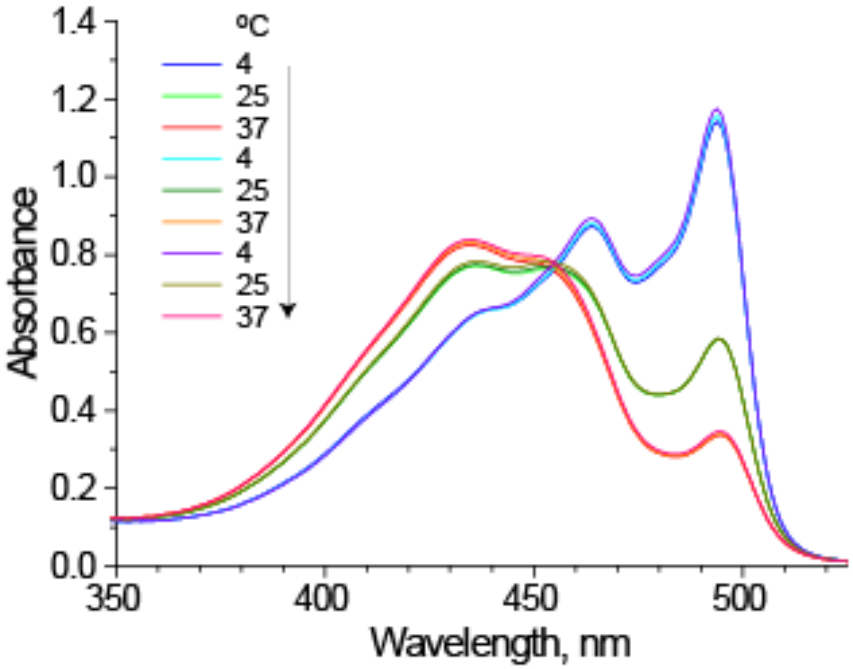
Ex 494 nm

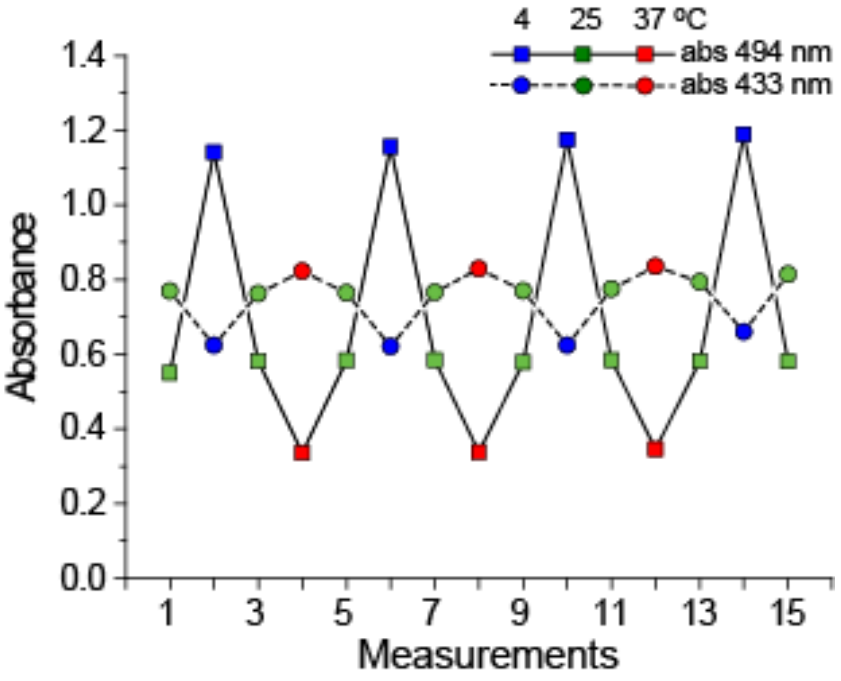
Em 505 nm



Sarkisyan el al., Sci. Rep. 2012

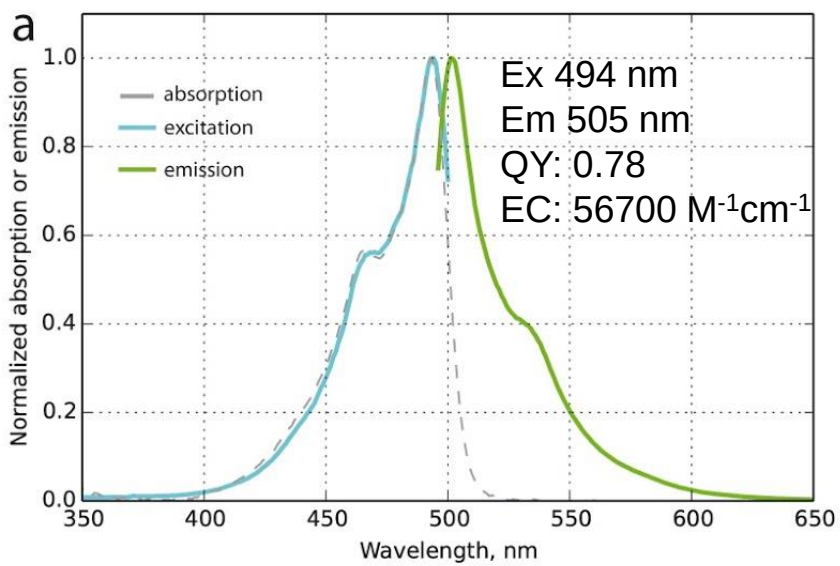
Temperature sensitivity of WasCFP

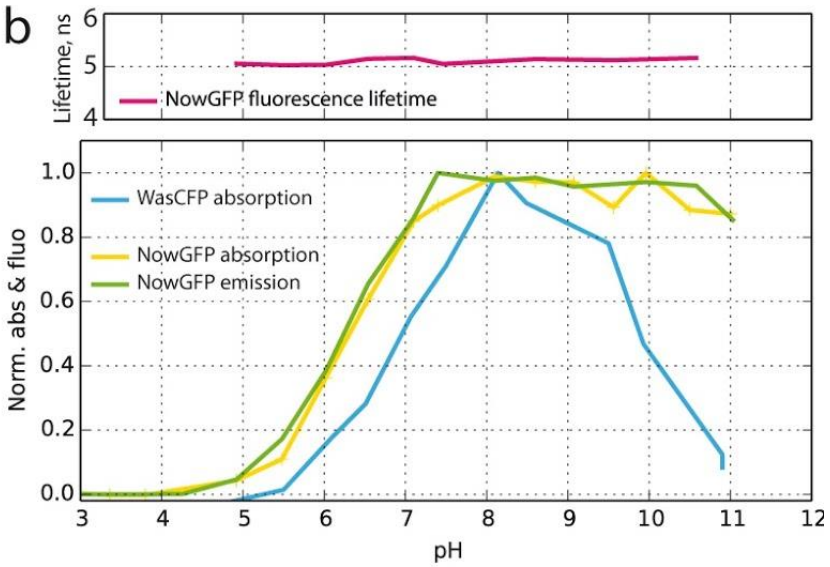


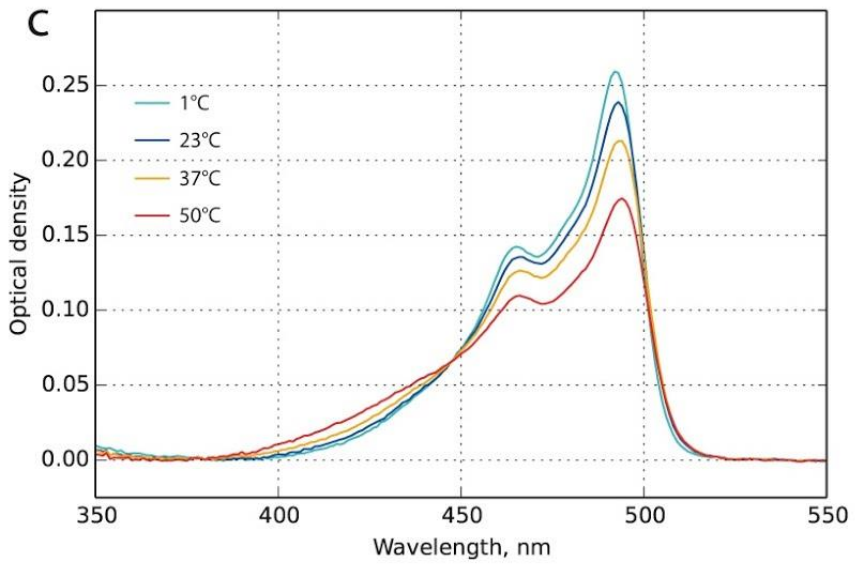


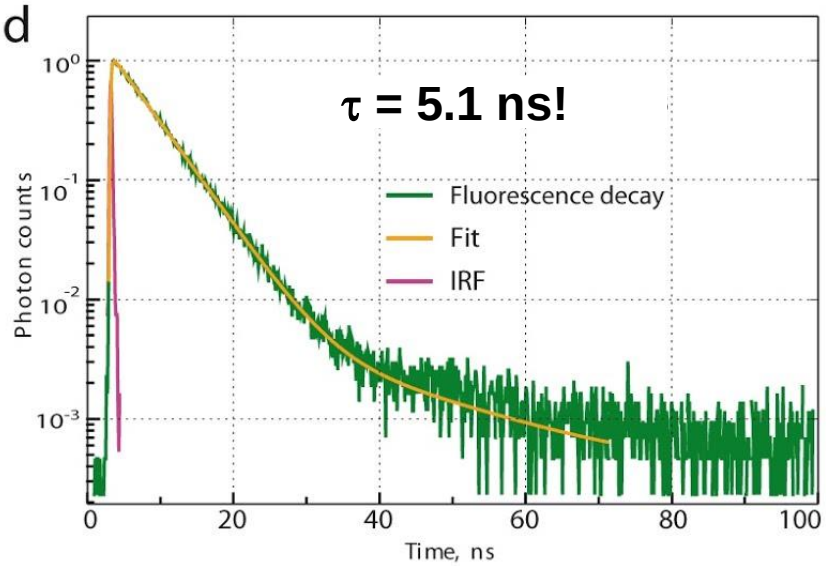
Sarkisyan el al., Sci. Rep. 2012

pH- and temperature-stable variant of WasCFP - NowGFP



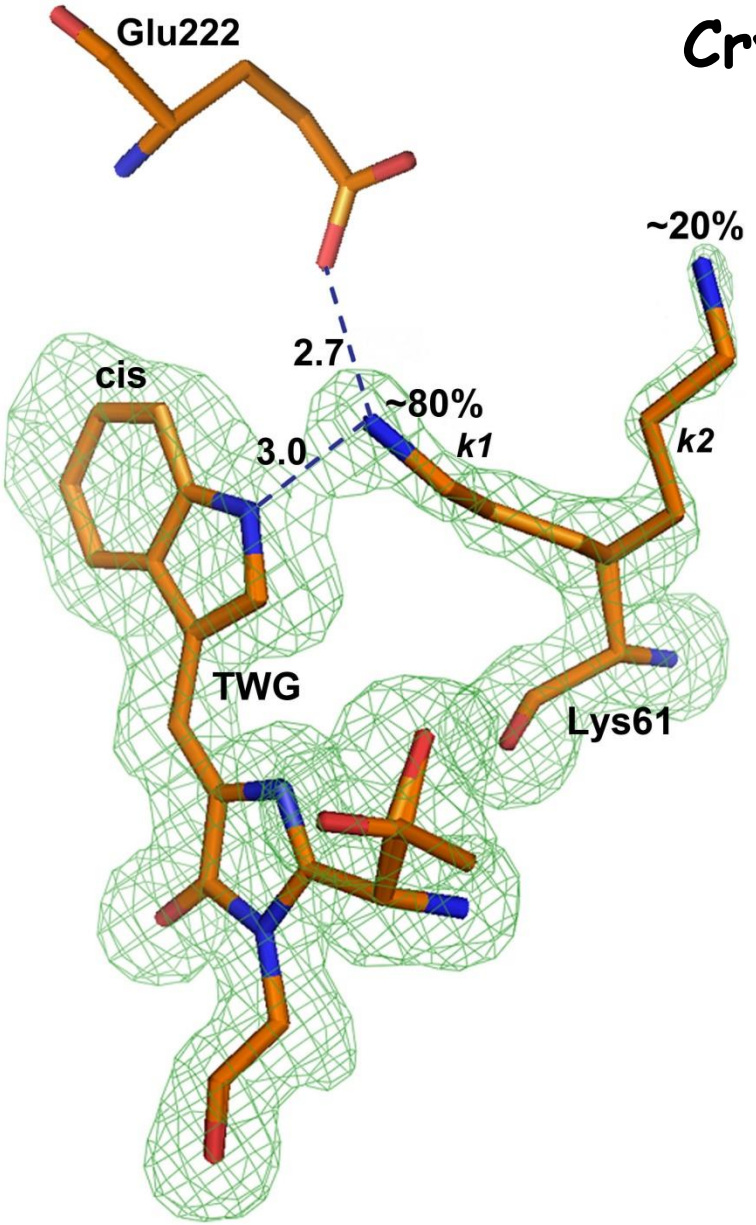






Sarkisyan et al., Biophys. J. 2015

Crystal structure of NowGFP

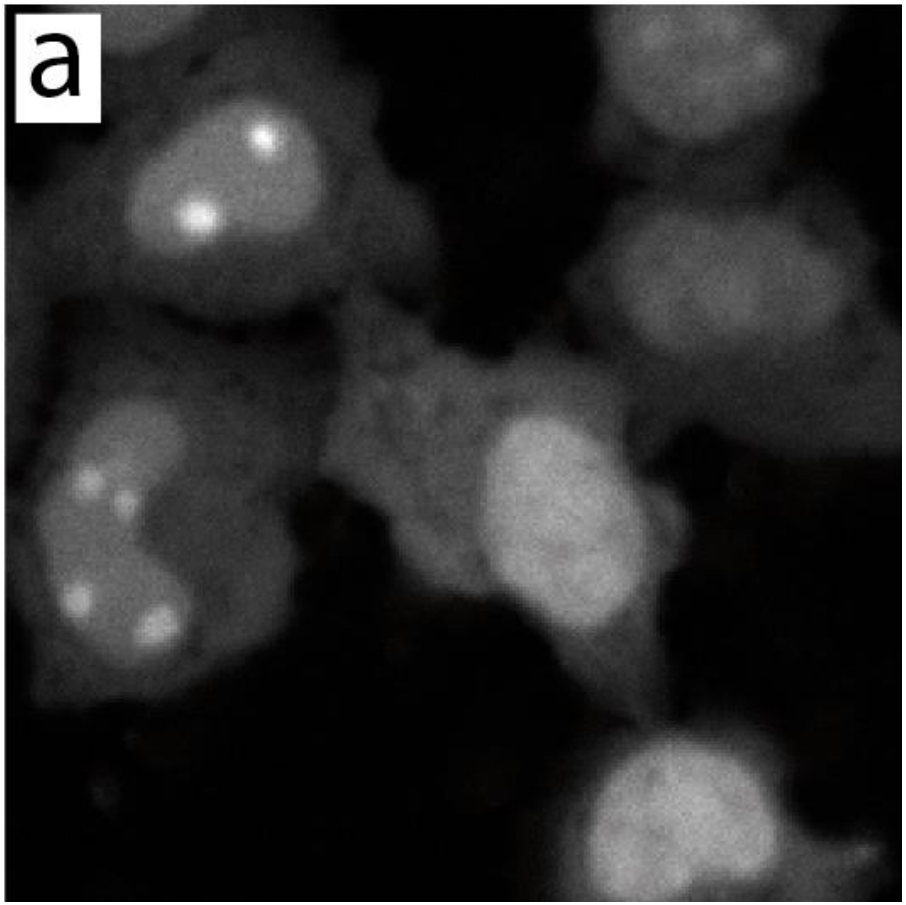


Lysine 61 is H-bonded with the chromophore!

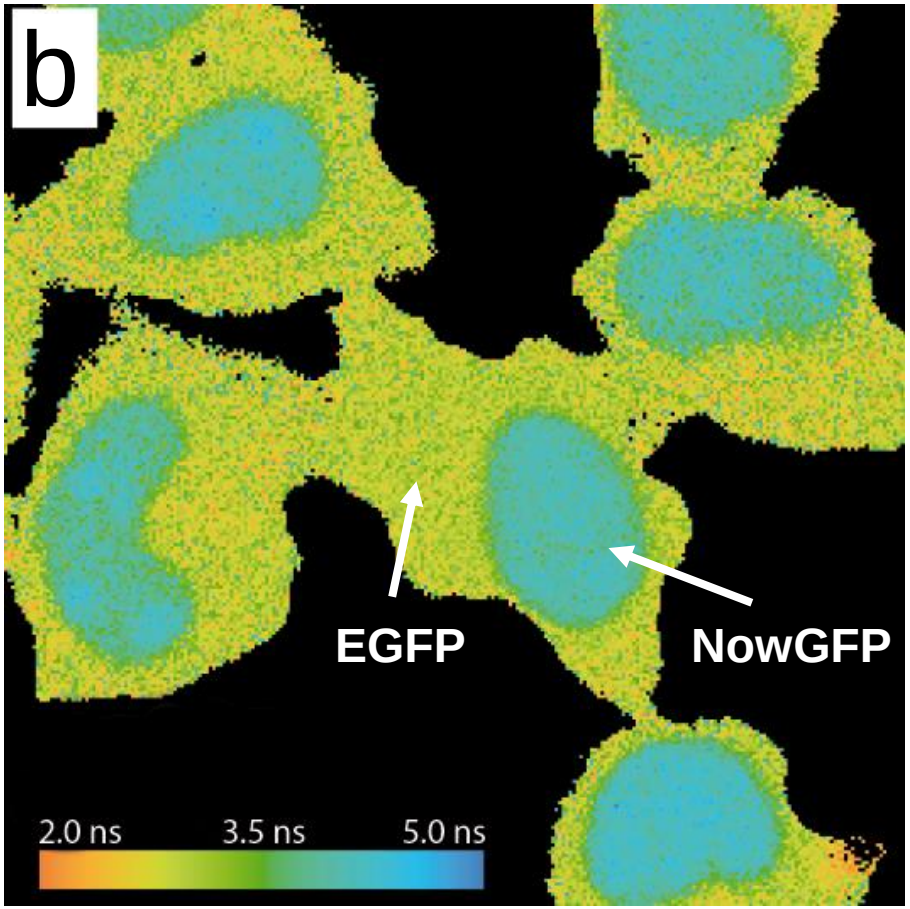
Pletnev et al., Acta Crystallogr D Biol Crystallogr. 2015

Fluorescence lifetime imaging (FLIM) with NowGFP:

"two-color" FLIM in green channel (EGFP + NowGFP)



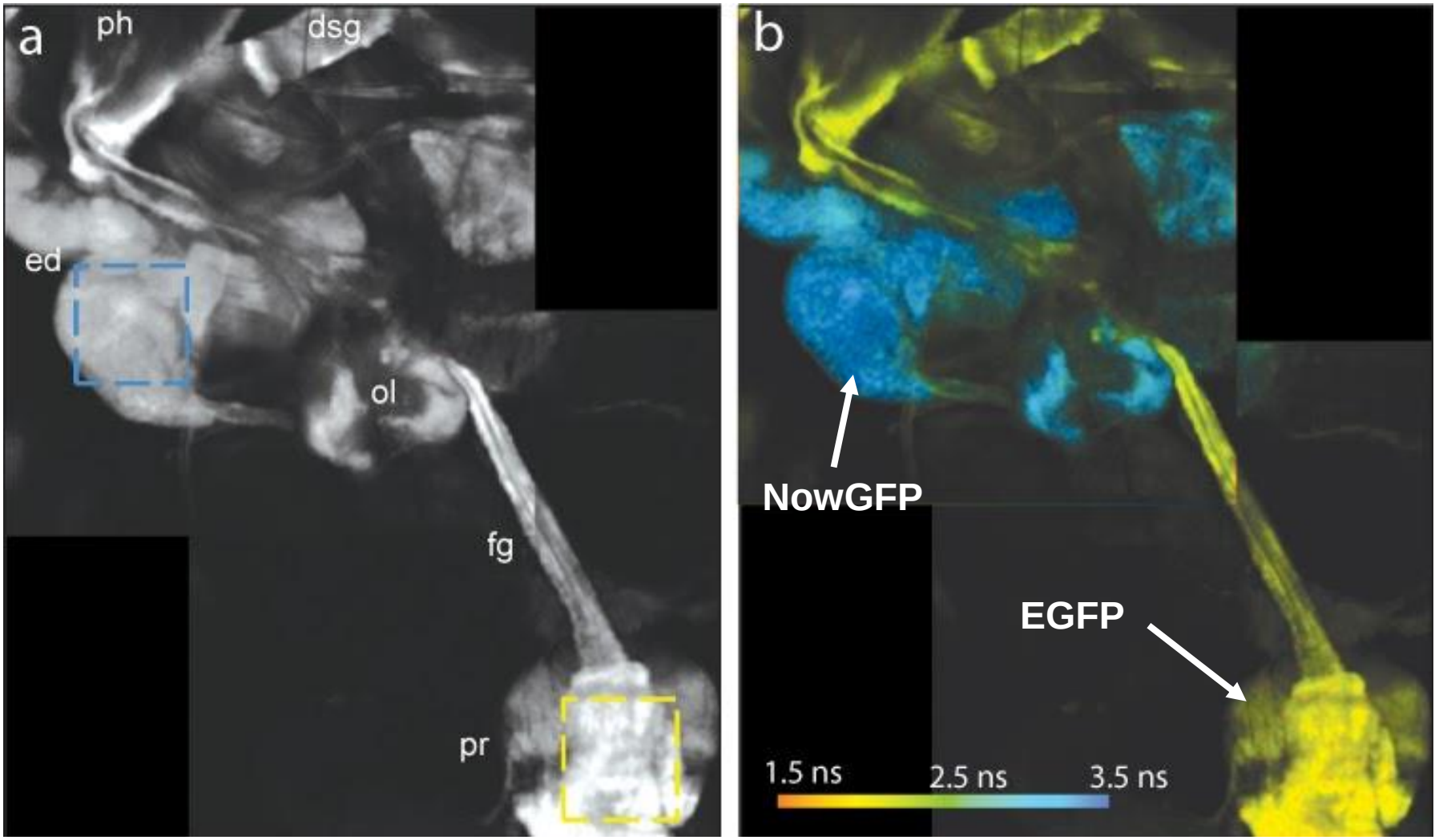
Intensity



FLIM

Sarkisyan et al., Biophys. J. 2015

"Two-color" FLIM in green channel (EGFP + NowGFP) in Drosophila larva

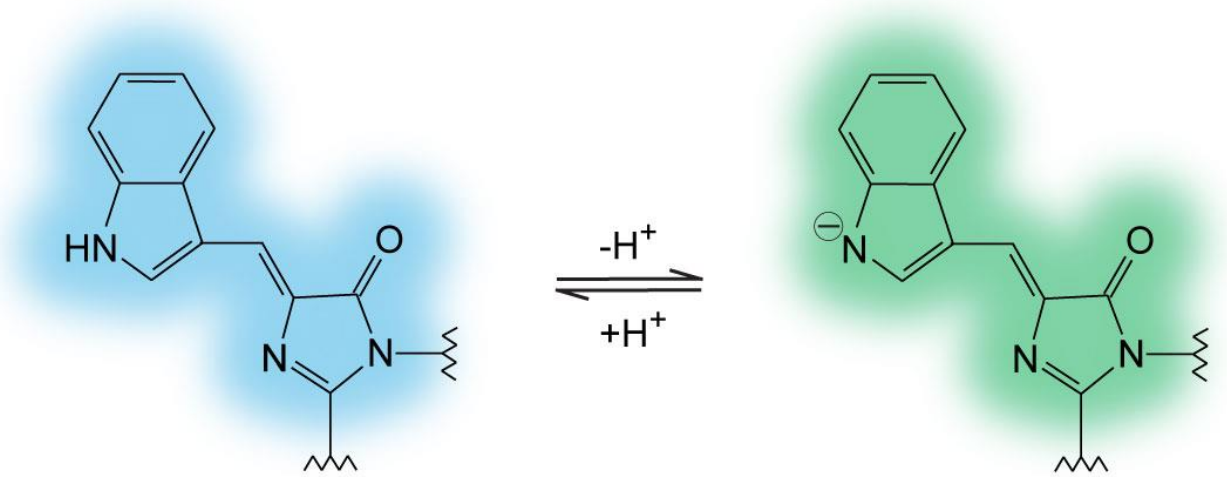


Intensity FLIM

Sarkisyan et al., Biophys. J. 2015

Perspectives:

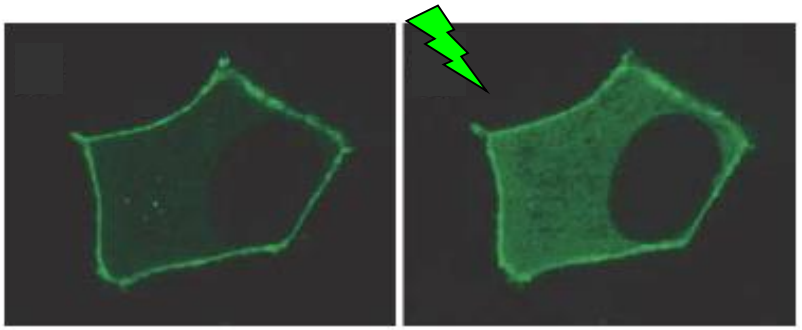
- Multiparameter FLIM
- Efficient FRET donors
- New sensors based on protonation-deprotonation of Trp-chromophore
- New red FPs with anionic Trp-chromophore
- New photoactivatable FPs with anionic Trp-chromophore



Fluorescent proteins as active photochemical agents

Genetically encoded photosensitizer KillerRed

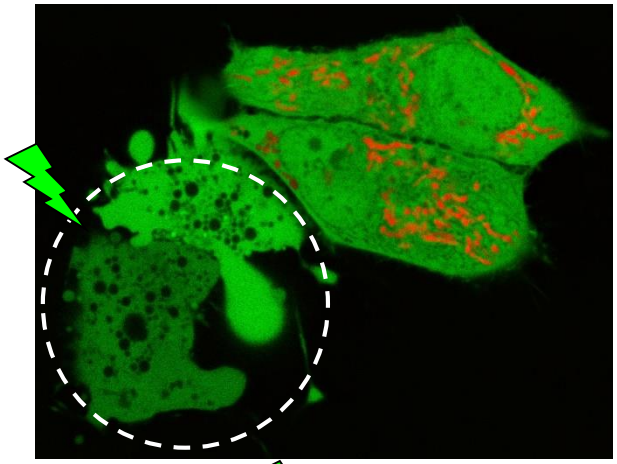
KillerRed-fusions – Inactivation of target proteins



KillerRed-membrane – Cell killing

KillerRed-mitochondria

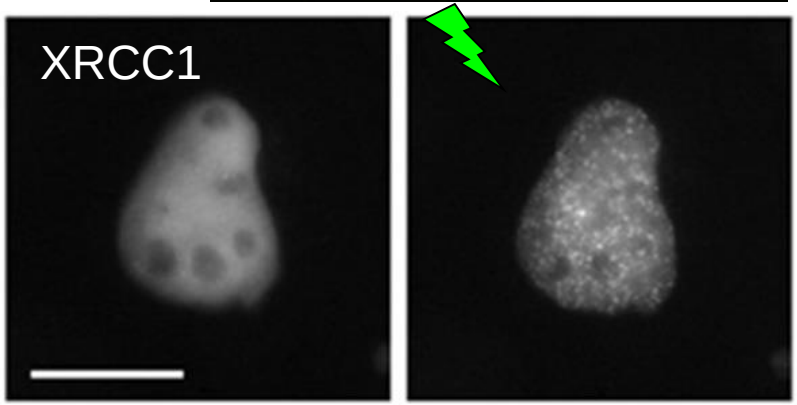
KillerRed-lysosome



KillerRed-H2B – DNA damage

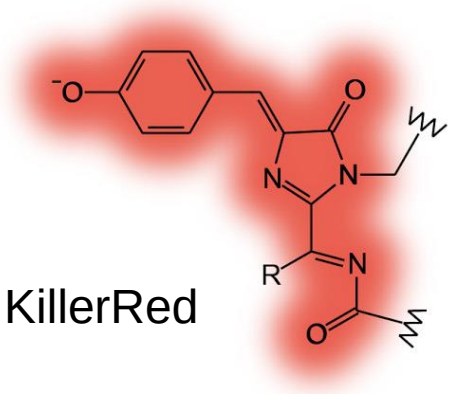
– Cell cycle arrest

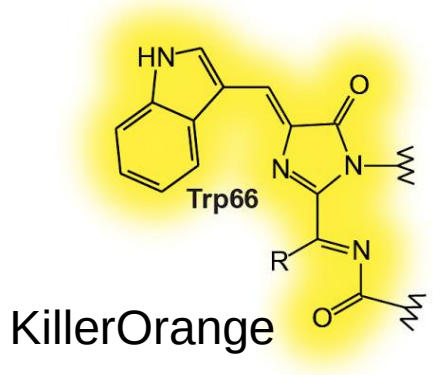
– Cell killing

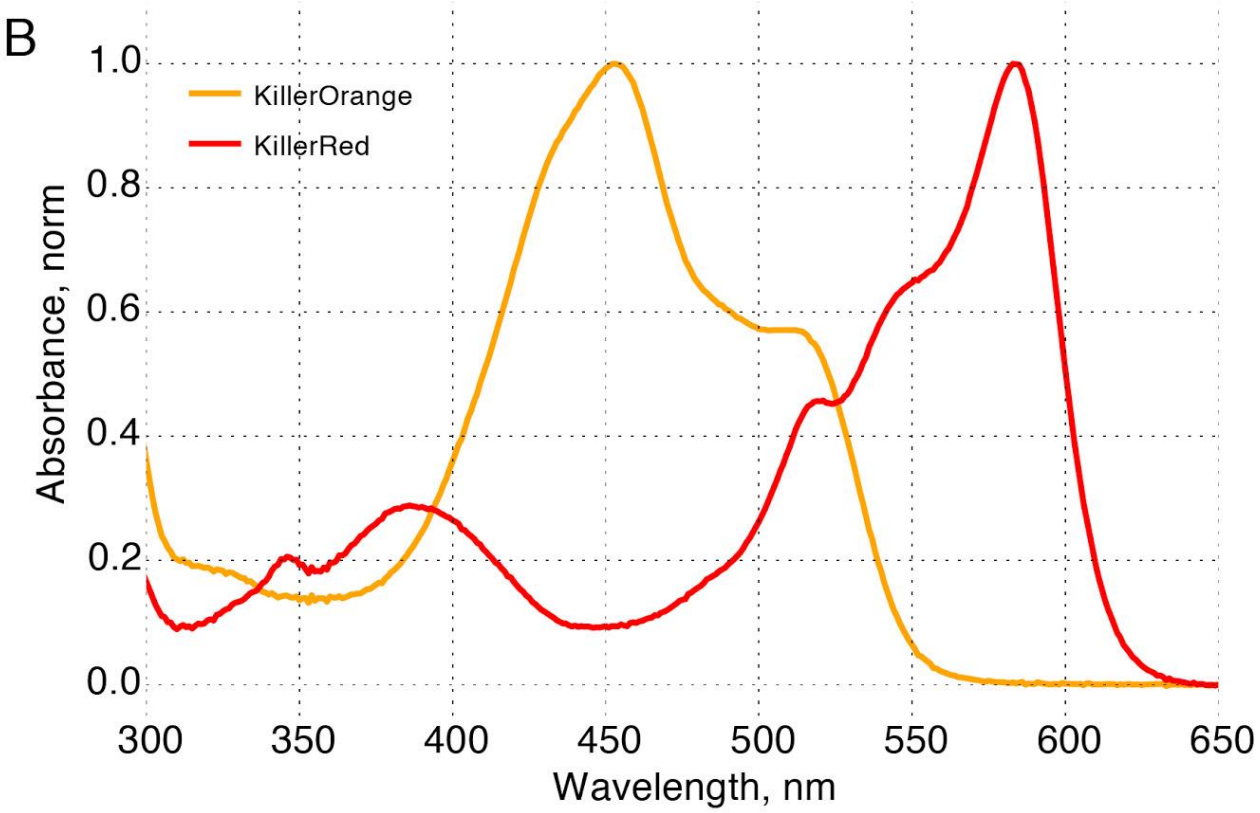


Bulina et al, Nat. Biotech. 2006
Serebrovskaya et al., Biochem. J., 2011
Serebrovskaya et al., J. Biophot. 2014

KillerOrange – a mutant of KillerRed

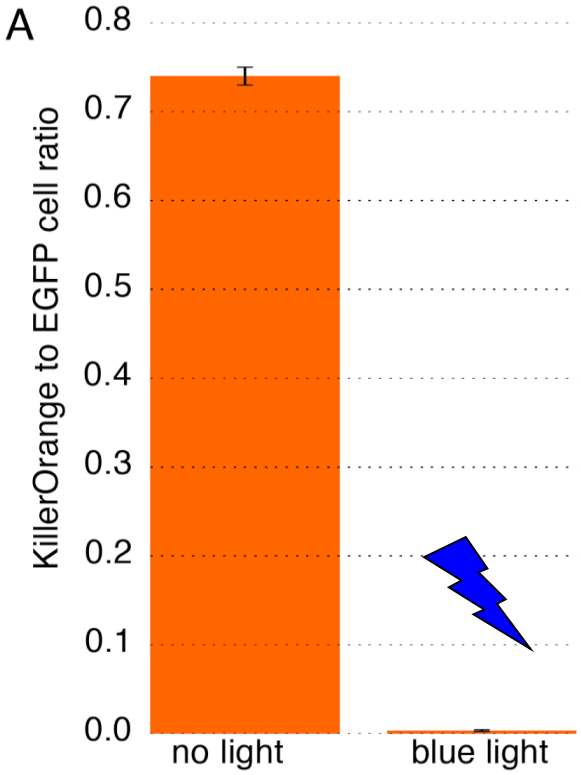


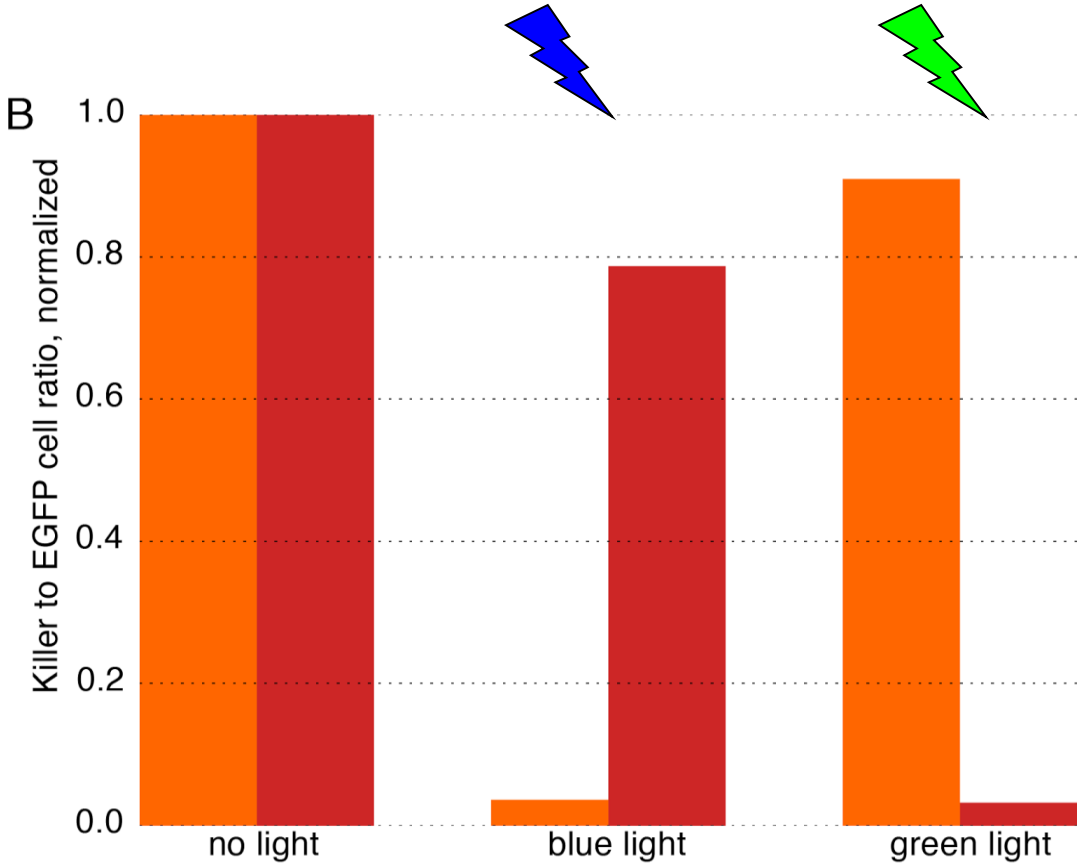




Sarkisyan et al PLOS1 2015

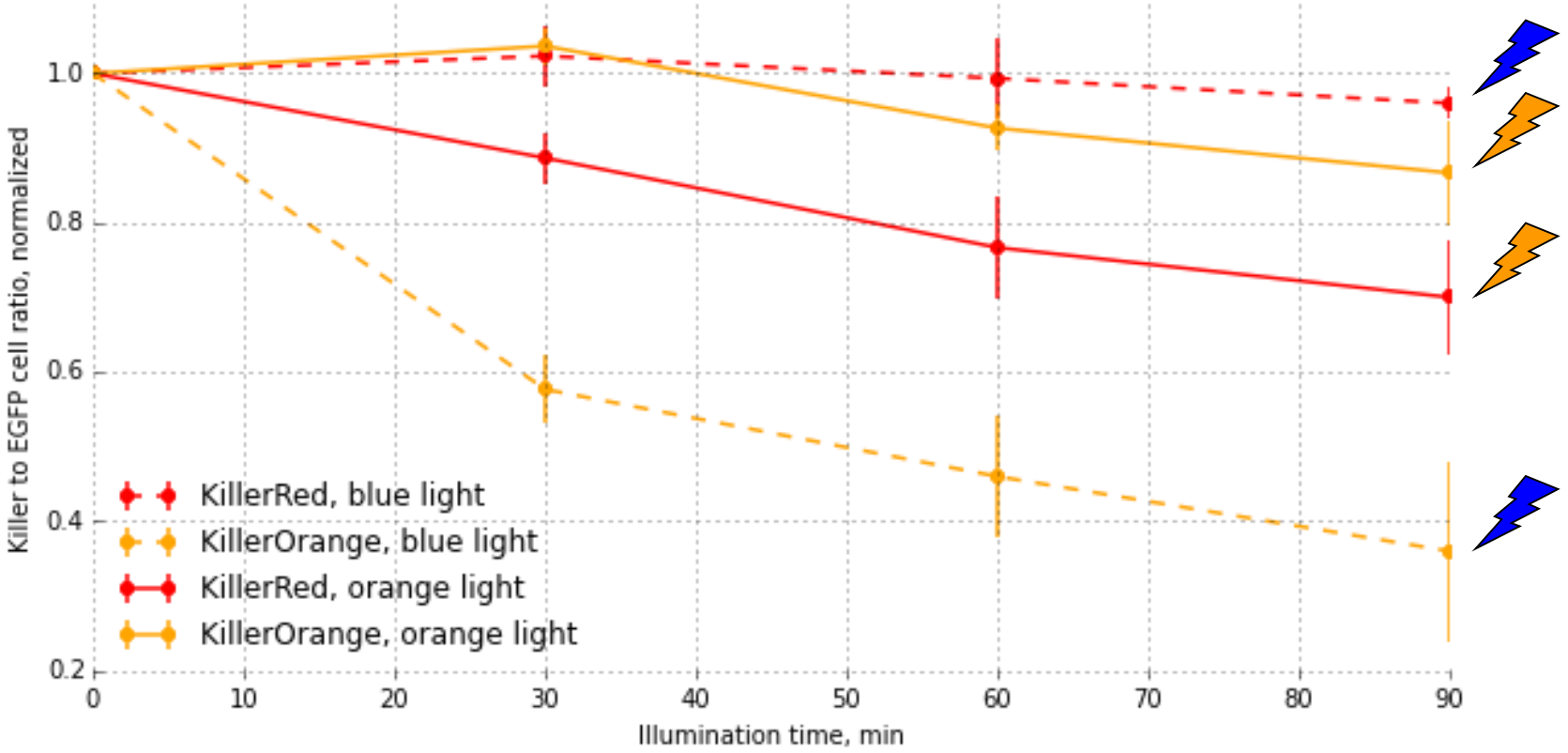
KillerOrange is phototoxic under blue light





Bacterial cells

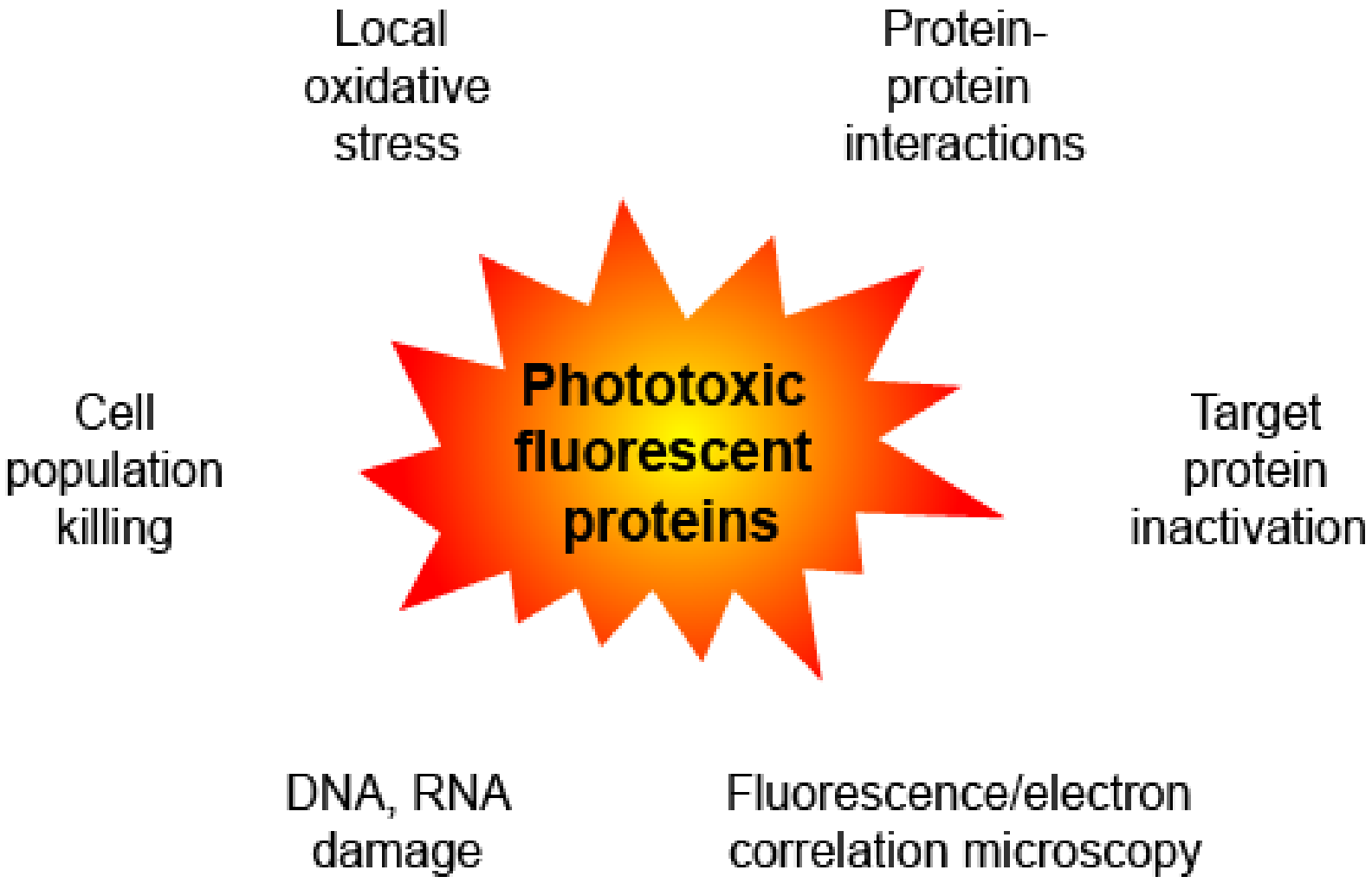
KillerOrange is phototoxic for mammalian cells



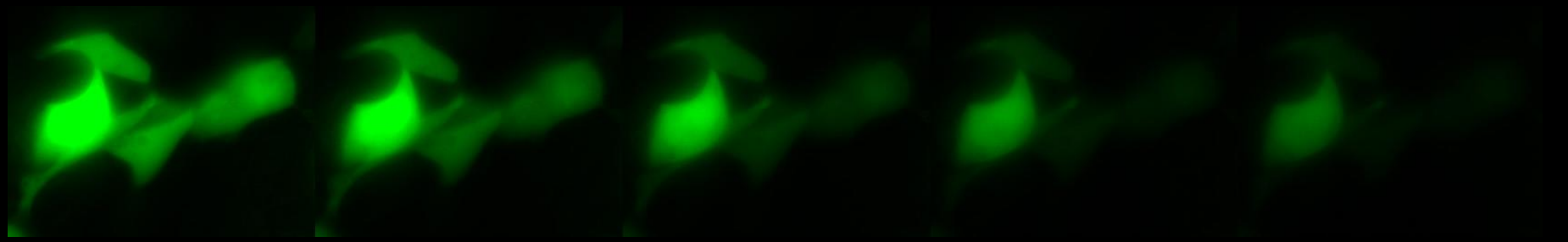
HeLa cells expressing KillerRed-mito or KillerOrange-mito.

Sarkisyan et al PLOS1 2015

Applications of phototoxic proteins

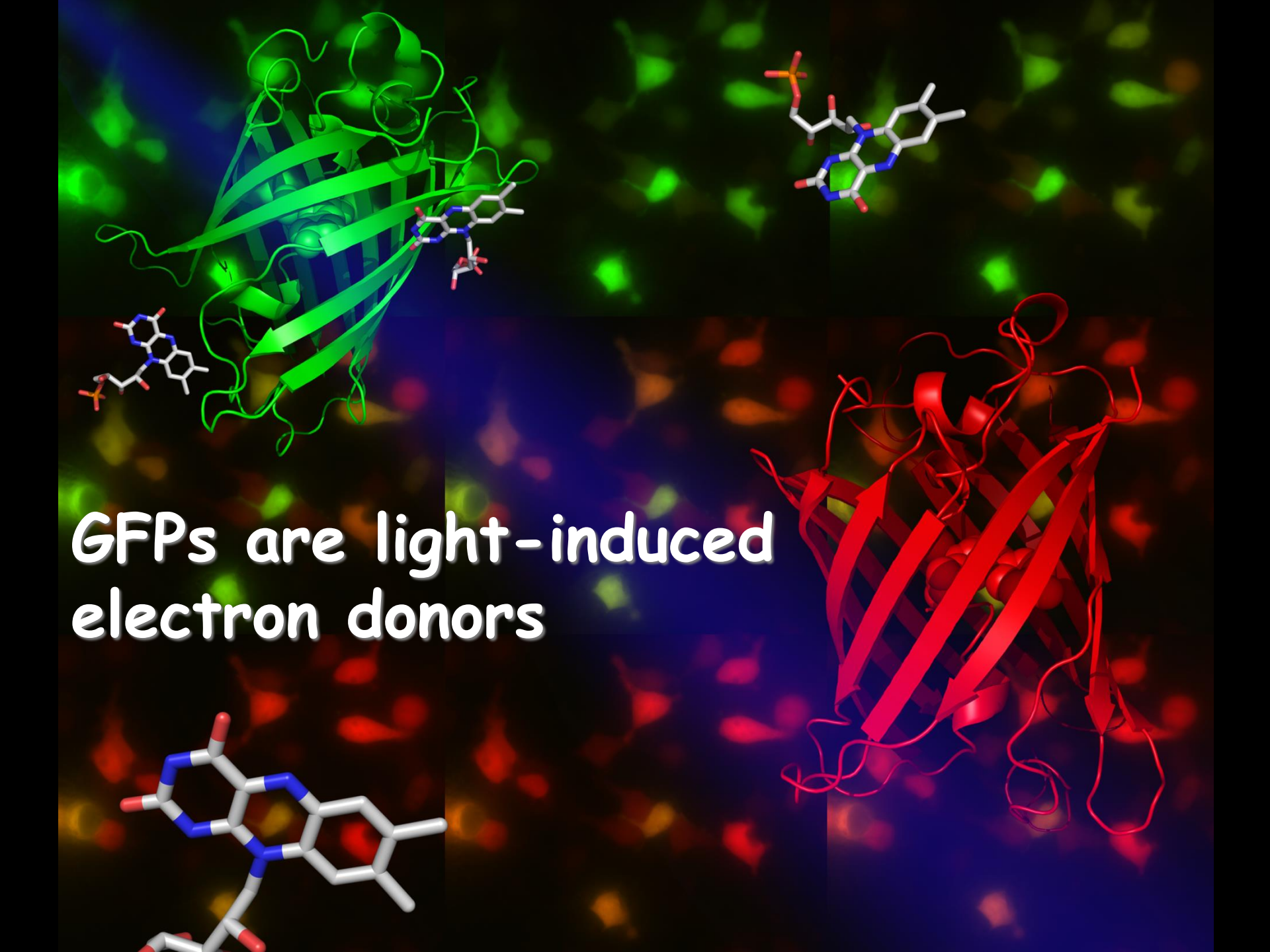


Photobleaching

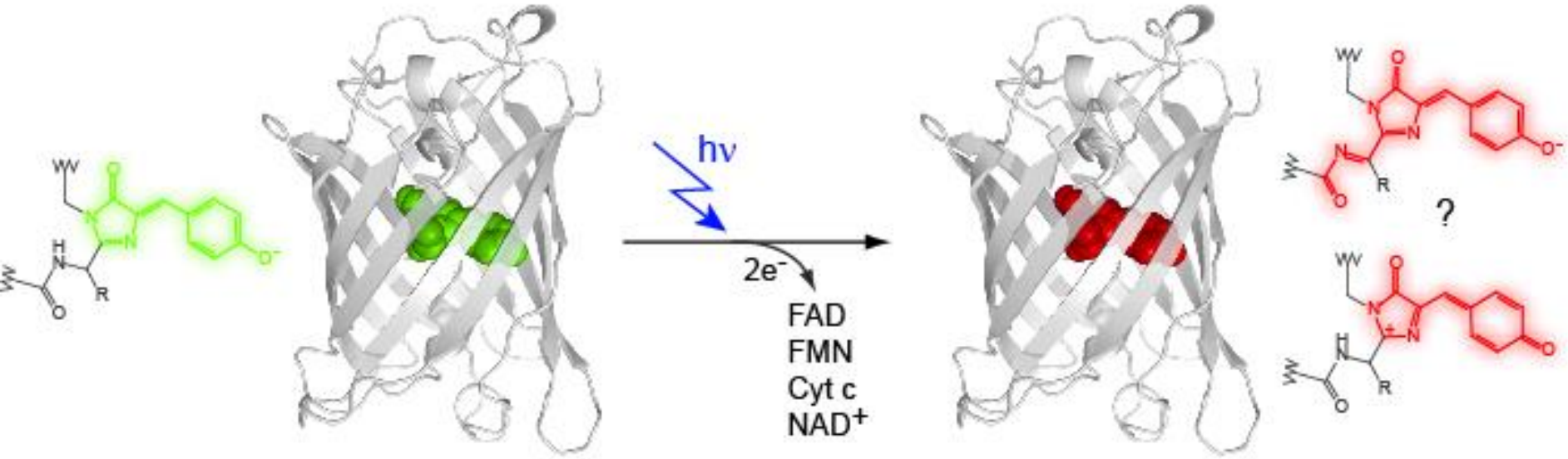


How to suppress photobleaching of fluorescent proteins in live cells?

- Improvement of hardware for microscopy (illumination regimes, sensitive detectors, etc)
- Improvement of fluorescent proteins by mutagenesis (e.g., EBFP2 is 550-fold more photostable than EBFP)
- Optimization of imaging media for live cell microscopy (“antifading reagents”)

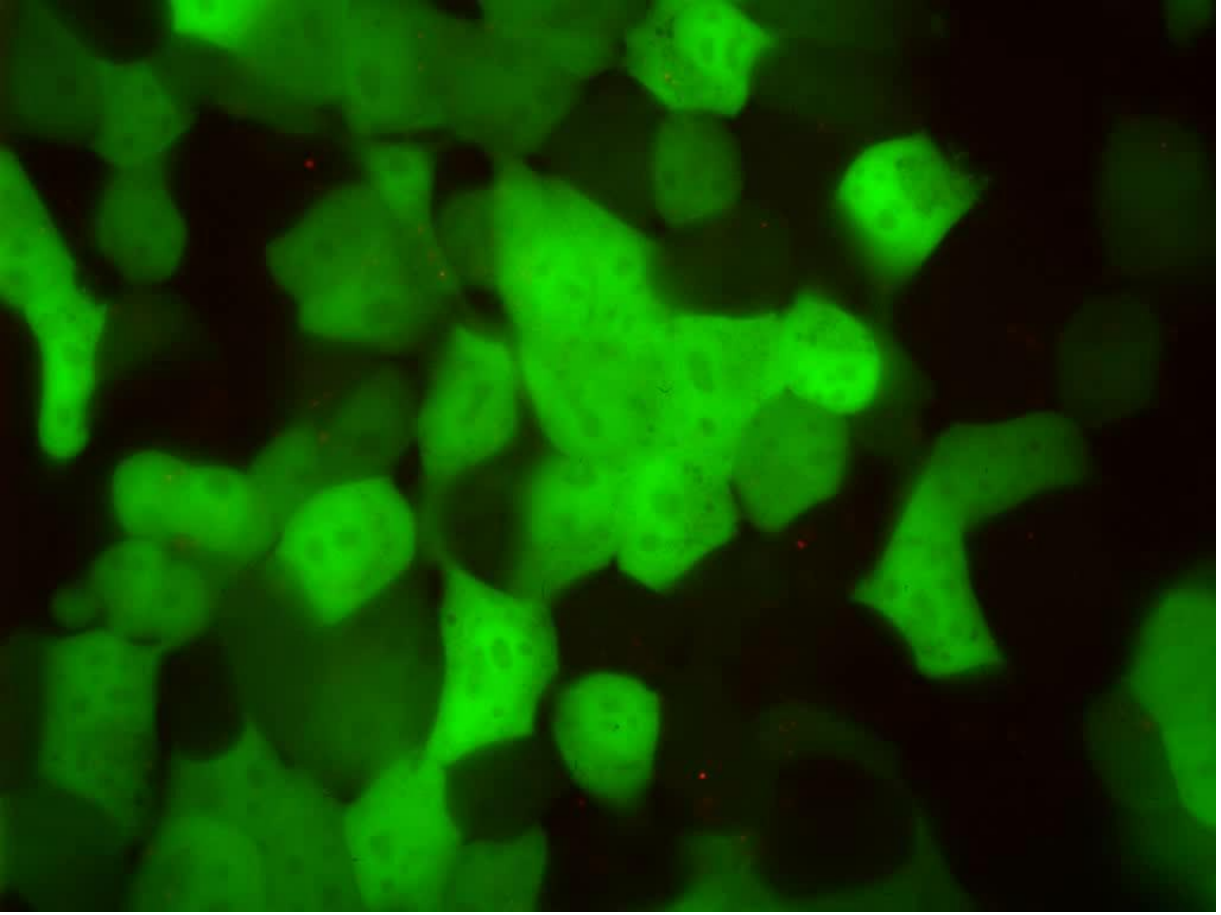

GFPs are light-induced
electron donors

Green-to-red photoconversion (oxidative redding) of EGFP

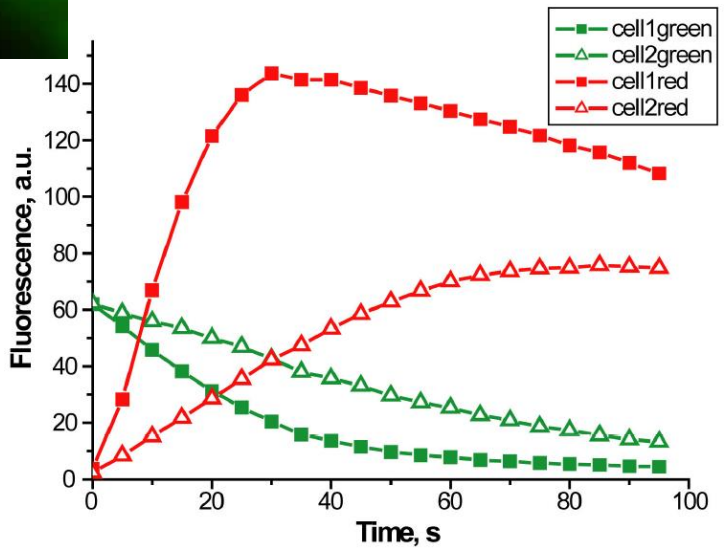


Bogdanov et al, Nature Chem. Biol. 2009

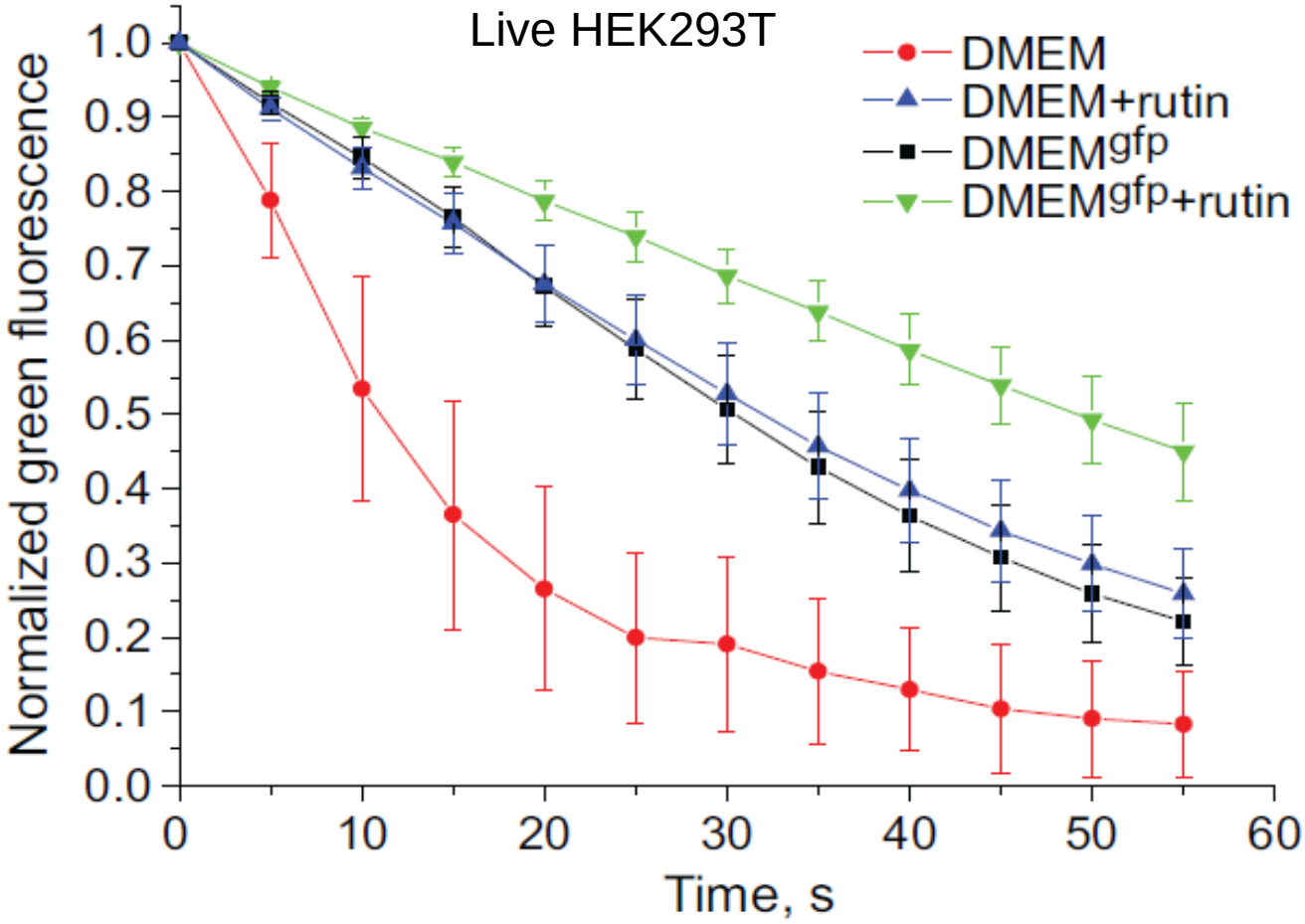
EGFP redding in mammalian cells



Live HEK293T cells in DMEM



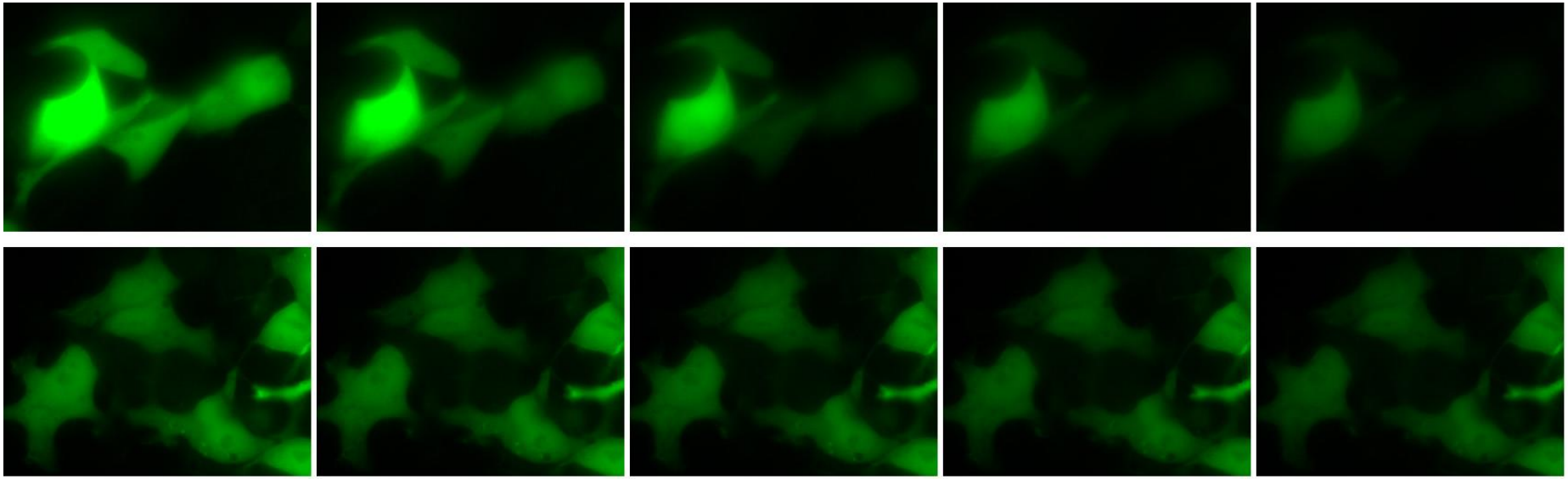
EGFP photostability can be enhanced by depletion of vitamits and addition of rutin



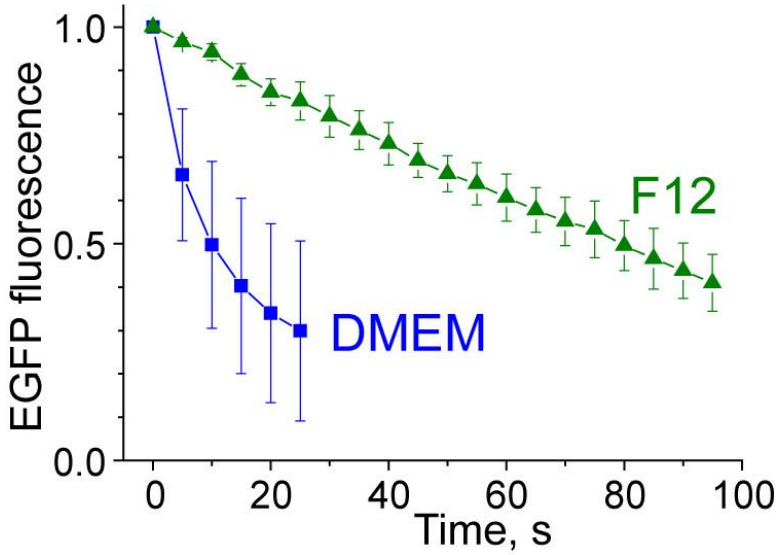
Bogdanov et al, Nat. Meth. 2009; PLOS One 2012

Imaging media for enhanced GFP photostability

DMEM



F12



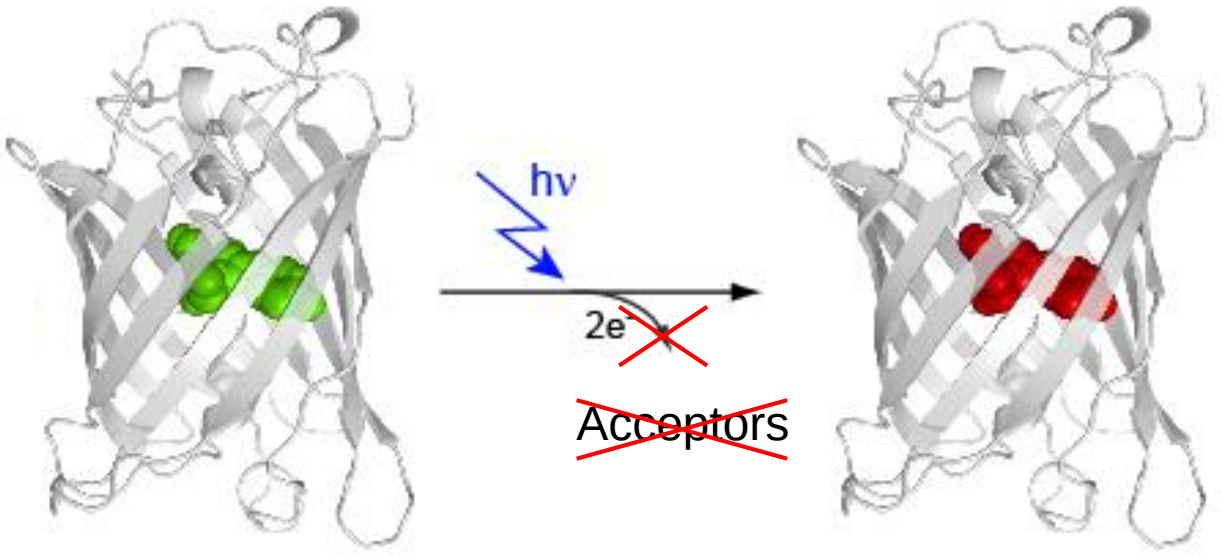
Mamontova et al.,
Biotechniques 2015

Suppression of oxidative redding of green FPs is an efficient way to improve photostability:

- Imaging media depleted of oxidants (flavin, piridoxal)

- Imaging media with antioxidants (rutin)

- Calculation of possible electron transfer pathways within FP; mutation of key residues



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